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THE TOXICITY OF COPPER TO CERTAIN NYMPHS OF THE EPHEMEROPTERA

by

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The toxic effects of copper to three species of British Mayfly nymphs were studied.These species were,Baetis rhodani,Ecdyonurus venosus and Rithrogena semicolorata.

The study was divided into the following sections :

I.Toxicity tests.These were carried out so that a basic profile of copper toxicity in respect to concentration could be arrived at for each species.Tests were also carried out to determine the effects of complicating factors such as temperature,pH and water hardness on the basic profiles of toxicity.Threshold of toxicity concentrations were also determined using the toxicity test technique.

It was found that B.rhodani is the most susceptible of the three species to copper,with R.semicolorata being the most tolerant.In all cases the extent of toxic effect bore a simple,direct relationship to the concentration of copper used.This toxic effect was made more acute by higher temperatures and low pH values,but was less acute in harder waters.

In this part of the study the results were expressed as Tlm survival curves and probit analyses.

A series of toxicity tests was also carried out to determine the effect of short exposure times to copper and it was found that complete recovery only occurs or is only possible in the case of low(less than 20mg/L copper sulphate)concentrations and exposures of less than six hours.

2.Serial sections of nymphs exposed to copper solutions were treated with Rubeanic acid which is a copper specific histological stain.This procedure identified areas of copper accumulation within the bodies of the nymphs. The distribution of copper was found to be as follows :

a. Within the lumen and cells of the mid-gut.
b. in parts of the central nervous system
c. in other organs including the Malpighian tubules and the gill filaments.

3. The effect of copper on the rate of respiration as measured by oxygen consumption was studied by using a sensitive micromanometer.

It was found that in the case of E.venosus there was very little effect but that in the case of the other two species copper depressed the rate of oxygen consumption.These results were related to the ecology of the respective species.

4. The rate of uptake of copper by the three species was measured using the radioactive isotope of copper,Cu64.Generally it was found that uptake is rapid up to sixteen hours.The fastest rate was for B.rhodani.An attempt was made to correlate these results with findings from earlier experiments in the study and some relationship was found to exist between the rate at which copper is taken up and the survival curves established by the toxicity tests in the first part of the study.

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CONTENTS

FIGURES

TABLES

PLATES

INTRODUCTION

The theoretical concepts underlying this study are fairly simple and stem from two main sources. The first is a result of man's industrial activities. Large industries, while undoubtedly providing certain material benefits for society, tend to be bad for the environment generally. This effect is usually due to the fact that as well as manufacturing desirable products these industries also manufacture highly undesirable waste products. In the case of copper such waste has its origins from those industries which use copper containing pickling liquors as well as those producing copper plating wastes and cupro-ammonium rayon wastes. Such unwanted byproducts can be disposed of by diluting, which often means that they are got rid of by depositing them into either freshwater systems or the sea. In many cases such effluent is untreated and can be very harmful to the ecosystem into which it is introduced.

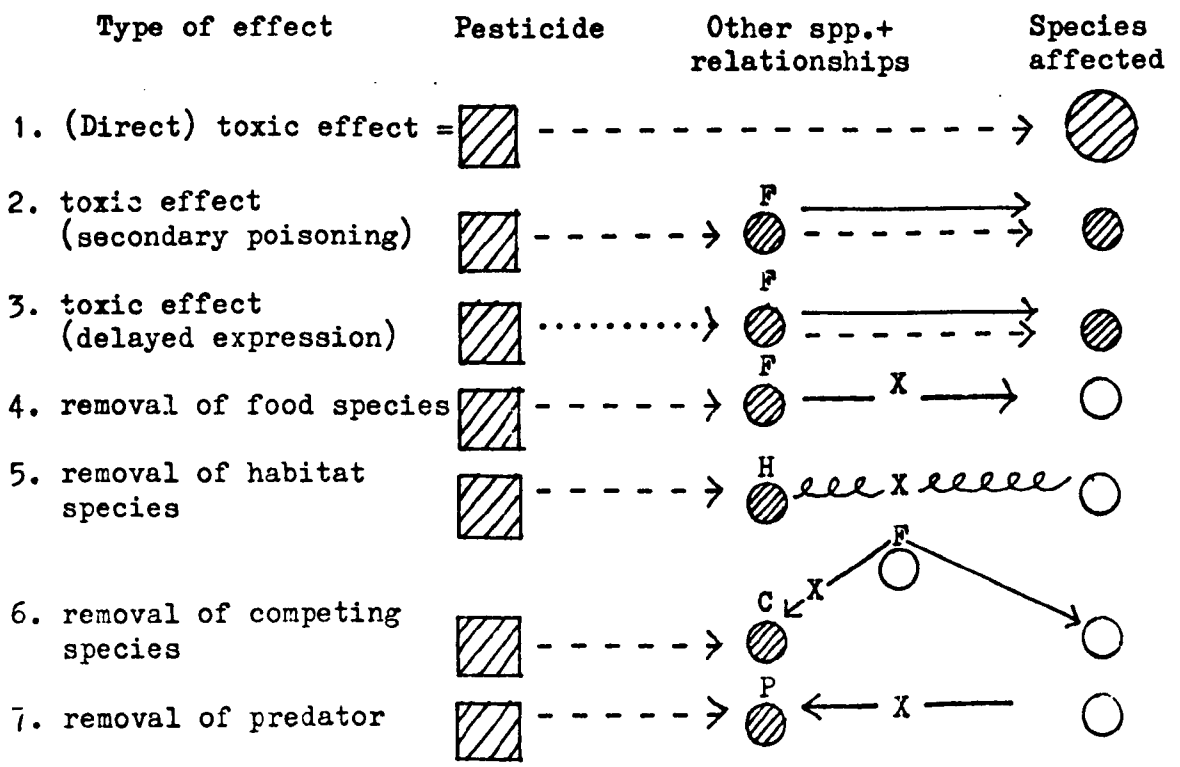
The second starting point for this study is also concerned with water contamination by copper but this time it is a more deliberate one, i.e. the use of copper in pesticides which are directed towards a single species in a given ecosystem. Pesticides can be defined as economic poisons which can be used to control a wide range of animals and plants and the history of copper being used as the toxic agent in such pesticides is a long one stretching back to ancient times. The more recent history of this use of copper is also well documented (MOORE and KELLERMAN, 1904; PRESCOTT, 1948; HASSALL, 1963; McBRIEN and HASSALL, 1965). Although most of the applications of copper have been in a marine environment copper has been and is being used in freshwater situations where it is

introduced in antialgicidal preparations. Here the pesticide can be applied by spraying, as is the case in the control of Potomageton and filamentous algae in freshwater systems. The problem with such procedures is that if the pesticide used is unselective, and copper preparations usually are, then there will be side effects on organisms other than those against which the pesticide was originally directed. These side effects may remain unnoticed if they affect organisms which we do not value such as the invertebrate fauna of a river or lake. This seems to have been the case in the past, since, although much work has been carried out in relation to the effects of certain toxicants on fish, which are commercially important, comparatively little time has been given to the study of the effects of these same toxicants on invertebrate animals which have no obvious commercial importance.

The situation can be rather complicated when one takes into account the sub-acute effects which pesticides may exert on the behaviour, growth, mortality, reproduction, pathology, resistance and genetics of exposed organisms. And the problem is even more intensified when one takes into account factors such as the extreme susceptibility of freshwater organisms, the complexity of freshwater environments and their variety.

At present it is difficult to comprehend the total effects of pesticides because there seems to be little knowledge concerned with how a wide variety of organisms will react to certain toxicants, however these are introduced into their environment. Certainly, little work seems to have been carried out using Mayflies as test

animals, and this study therefore attempts to fill one of the many gaps that exist in present knowledge concerning the effects of toxicants on the invertebrate freshwater fauna. Before discussing this group of insects specifically, however, it is worthwhile to remember that, as MOORE (1967) has pointed out, each application of a pesticide will result in a pesticide ecosystem, even if it is aimed at one species. In other words, the application of a copper containing pesticide or effluent can have a toxic effect on a wide range of organisms occupying different trophic levels within that ecosystem. The realization of these far-reaching effects has come with a greater understanding of the ecological relationships which exist between the species occupying an ecosystem and there are several ways in which a toxicant can exert an effect on a species other than the one which it is supposed to be controlling. MOORE (1967) represented these pathways as follows:



Key: ⟶ food relationships (arrow points to consumer)

habitat relationship

- - → harmful toxicological effect of pesticide

X ecological relationship broken by pesticide

.....→ harmless effect of pesticide

 pesticide applied

 pesticide residue

C = competing species

F = food species

H = habitat species

P = predator species

As can be seen from this scheme, a toxicant does not affect a single species at all but can either directly or indirectly exert an effect on a wide range of species. Clearly, some knowledge concerning how a toxicant is likely to affect a given species other than that against which it was employed would be desirable. In a later section this interrelationship which exists between the organisms of an ecosystem is stressed again with emphasis on the mayfly nymphs, so it is only mentioned in passing here that it is generally accepted that the many species of Ephemeroptera form a quantitatively important part of the invertebrate fauna of rivers and streams. Also they have been found to be useful as indicators of oxygen conditions in running waters as well as having an important role to play as the food of many types of fish (NAGELL, 1973). This group of insects is therefore of some general importance and some information regarding their reaction to a fairly common pollutant such as copper would seem to be desirable. Some study has been made of the effects of certain toxic materials on certain species of mayfly nymph (MUIRHEAD-THOMSON, 1973), but in such studies the toxicants

tested were derived mainly from agricultural pesticides which contained no copper. The primary aim of this study is, therefore, to study the toxicity of copper in terms of MOORE's first classification, i.e. the direct effect of copper on certain species of ephemeropteran nymphs. Although the ecological implications of such effects should not be lost sight of, this is a purely laboratory investigation in which the toxic effects of copper to three species of Mayfly nymph are studied in controlled laboratory conditions employing several techniques.

The methods used for this investigation fall into four areas which can be summarized as follows:

1. Toxicity tests.
2. Histochemistry.
3. Effect of copper on a basic physiological process: respiration.
4. Radioisotope studies to measure rate of uptake.

The toxicity test was used as a useful starting point aimed at giving a basic profile of the way in which the nymphs react to copper when subjected to certain variables such as temperature, pH and concentration. The variables chosen are those which are likely to be complicating factors in the 'natural' environment so that the data obtained from these tests could perhaps be of use in predicting to some degree how the Mayfly section of the invertebrate fauna of a river is likely to react to copper in certain situations. For example, after having established a basic pattern of reaction to copper it would be useful to know how this is affected by changes in

temperature such as will result in a river into which cooling waters are introduced from a nearby factory. Also, many effluents discharged into river systems will alter the pH of the water and, again, it may be of use to know how this will affect the susceptibility of the Mayfly population to copper.

Having established the degree of poisoning of copper the next, and perhaps most important, step would seem to be to make some attempt in discovering how this poisoning is brought about. Here it is important firstly to distinguish between an external, surface effect and an internal physiological one. In the case of heavy metal poisoning to fish it has been found that the toxic effect is due to the combination of the heavy metal ion with the mucus of the gill to form an impermeable ion/mucus complex which does not allow gaseous exchange to take place at the gill surfaces. The fish were therefore being asphyxiated (WESTFALL, 1945). This type of effect can be distinguished from a true physiological one since it is due to a physical barrier blocking the gill system of the fish. (This distinction is, perhaps, not altogether a valid one since all physiological processes are ultimately due to physical and chemical mechanisms.)

As a preliminary step towards arriving at some conclusion on this point with regard to Mayfly nymphs it would seem necessary to establish that copper is in fact entering the animals in the first place. In the nymphs of the Ephemeroptera the cuticle is comparatively thick and impervious and the cuticle of the tracheal gills has the same permeability as that of the rest of the cuticle (SHAW and STOBBART, 1963). If copper is being taken in, therefore,

it is not likely to be percutaneously but rather orally by drinking or during eating, which will also result in the ingestion of small amounts of water. If this is so then it should be possible to detect copper in the gut of nymphs. This would give direct evidence of the mode, or at least one of the modes, of entry of copper.

Once copper is seen to have actually entered the gut it is reasonable to suppose that it can pass through the gut wall and be, to some degree, assimilated into the body cells where it can exert a physiological effect on certain systems. Respiration can, perhaps, be regarded as a basic unit of metabolism and one which is comparatively simple to study, and therefore this is chosen for investigation here in an attempt to detect any effects that copper may have on it. A further reason for regarding respiration as a system likely to give easily detectable results is that copper has for some time now been associated with a certain catalytic role in respiration and oxygen uptake in a variety of bloods (see chapter on biochemistry of copper). It may be possible, therefore, that metabolic pathways exist whereby copper tends to be accumulated at the sites of respiration within cells (mitochondria) and it is here that the effects of excess amounts of this ion may show themselves first.

As a consequence of finding evidence that copper is indeed being taken up by the nymphs it would seem necessary to find the rate at which this uptake is taking place, since then it would be possible to predict with greater certainty and accuracy the likely effects of

copper over a given period of time. Also, studies on the uptake rate could throw some light on the question of active copper excretion in Mayfly nymphs; this information could then act as a springboard for further extensive studies in which the detailed patterns of uptake under different conditions could be found, giving a more accurate overall picture of copper toxicity than that supplied by toxicity tests alone.

Chapter 1. REVIEW OF LITERATURE

THE TOXICITY OF COPPER TO CERTAIN EPHEMEROPTERID NYMPHS

In 1894 the physiologists RINGER and SAINSBURY were carrying out a series of experiments concerned with the action of sodium, potassium and calcium salts on the freshwater worm, Tubifex rivulorum. For this investigation they set up a parallel series of experiments using certain salts of the metals under investigation, in solution, in both tap and distilled water. During the course of these experiments it became clear that there were two very different types of action on the test animals in these parallel tests. In the tap water series Tubifex lived for an indefinite period in the control beakers. If some degree of disintegration occurred it was only in a small percentage of the animals and did not occur until late in the experiment, i.e. after a period some days had elapsed, and even this was attributed to the effects of mechanical damage suffered by some of the animals. In the distilled water series, on the other hand, the results contrasted dramatically. Here the animals were affected much more drastically, this effect being described at the time as follows: "In this medium the organisms continue their active movements but gradually disintegration appears and ultimately they are all converted into a globular debris".

In the following year an explanation for these observations

was offered. This was put forward by the biologist LOCKE, who was working both with tadpoles and Tubifex. LOCKE found that "tadpoles placed in distilled water rapidly became motionless after a period varying as a rule between two and six hours, paralysis is quickly followed by disintegration, indeed signs of desquamination may be observed before movement has entirely ceased". LOCKE obtained similar results by immersing Tubifex in distilled water. These and similar observations were made by LOCKE during the course of a study which he in fact began in 1883 which was concerned with a comparison between the action of a 0.6% sodium chloride solution and a 0.6% solution of sodium chloride which contained a small percentage of sodium oxalate. LOCKE made up these solutions in distilled water and found that, "The 0.6 sodium chloride solutions had a much worse influence on the persistence of good contractility of the grass-frog's sartorius than I had previously observed such a solution to have". This effect was not due to the absence of certain salts (e.g. calcium, magnesium, potassium) since he added these and found that solutions with these additives (again made up in distilled water) did not approach in any way "the preservative and recuperative action on voluntary muscle of a 0.6% sodium chloride solution made up in tap water". The distilled water, therefore, seemed to be at the root of the problem. On analysis of the various distilled waters which were available to him, LOCKE attributed this "poisonous action" to the presence of very minute traces of compounds of heavy metals which his analyses showed to be dissolved in these distilled waters. Further, he found that water which did not possess this

toxicity (i.e. tap water) became possessed of it after being allowed to stand in contact with metallic copper, silver, tin, iron and mercury.

LOCKE gave the term "oligodynamik" to this effect of minute traces of certain heavy metals in water. To obtain water not possessing oligodynamik properties (neutral water in LOCKE's terminology) he found it necessary to distill water in glass apparatus. It appears, therefore, that the vast field of heavy metal toxicity was opened up only incidentally to other, initially unrelated studies, attention being drawn to it by the fact that water used to be distilled in stills made of copper and tin.

In the paper which LOCKE published in 1895 he goes on to describe a series of experiments which he carried out using Tubifex and frog tadpoles in which he uses waters distilled in both copper and glass stills. He found that water from the glass stills which had been stored in glass vessels had no "noxious action on tadpoles that could be observed, and by no means a rapid one on Tubifex". The water prepared in copper stills, however, was found to be toxic to these organisms. Of the metals that LOCKE went on to investigate he found that copper was the most active in "rendering water unable to support the life of Tubifex and tadpoles". Further, he found that water which had remained in contact with a strip of copper exposing 36 sq.in. of surface for 26 hours could not support the life of Tubifex or tadpoles overnight (16 hours). This observation records perhaps the first toxicity test to be carried out with copper and, if the above observations are accurate, the toxicity of this metal is seen to have been appreciated at an early stage in

the history of work on heavy metal toxicity.

It was also at about this time that the phenomenon of antagonism was first noted. In 1893 NAEGELI, who, like LOCKE, had found that water distilled in copper stills was lethal to Tubifex made the discovery that this property could be dispelled by the addition of charcoal, sulphur, paraffin and other amorphous substances to the water. This property displayed by certain substances of counteracting the effect of the toxic substance, i.e. reducing or completely neutralizing its toxic properties, is the basis of antagonism. More recently three general types of antagonism have come to be recognized - that observed by NAEGELI belongs to the first category, i.e. the toxicity of a dissolved substance may be reduced on the addition of a second substance, not in itself toxic or even soluble. Since a fuller discussion on antagonism will follow later this subject will be left now in order to pursue a chronological survey.

Since these early studies on the toxicity of heavy metals there has been a tendency to use fish as test animals. One study made in 1900 by WARREN was, however, made on the effect of sodium chloride on Daphnia magna. Here a relationship was pointed to between the concentration of the solutions and the time which they took actually to kill the animals. This relationship was expressed by a graph which WARREN called the "survival curve". He found that between the concentrations of 0.9 and 6.0% this curve was a rectangular hyperbola. Below and above these concentrations the results were "indefinite". The introduction of the term "survival curve" in this paper is significant as it marks the start of a more

quantitative handling of data obtained from the increasingly precise and controlled toxicity tests. A pattern of procedure was therefore carried out which is still, fundamentally, the same today, i.e. a toxicity test followed by a quantitative processing of data which may take several related forms.

In the years that followed the survival curve was to assume an importance in the interpretation of toxicity test data which is still apparent. OSTWALD (1905) working on the toxicity of seawater to Gammarus pulex also obtained a curve the middle portion of which was a rectangular hyperbola. PITTENGER and VANDERKLEED (1915) working with goldfish concluded that in all cases the survival curve of an aquatic organism in a toxic solution should be a rectangular hyperbola, i.e. the concentration plotted against the reciprocal of the survival time should give a straight line. This line they called the "fatality curve".

By this time the rectangular hyperbola was beginning to be accepted as a standard result of toxicity tests and was perhaps also beginning to assume the status of a natural, immutable law in the minds of workers in this field. This outlook was no doubt strengthened when in 1917 CLAYBERG, working on the toxicity of aqueous solutions of chloroform and ether to the Sunfish, also concluded that the right shape for a survival curve to assume was that of a rectangular hyperbola. However, in the same year POWERS made a more comprehensive study of toxicity using a large range of salts, acids, alkalis and drugs, using the goldfish as his test animal. From these studies he concluded that in all cases the middle portion only of the fatality line or curve was straight. POWERS appears to

have been the first worker to observe that for all toxic substances there is a more or less well defined lower limit of concentration below which the survival time becomes indefinite and protracted. To this he gave the term "threshold concentration". It is true that WARREN also observed this in 1900 but he does not seem to have stressed the significance of this and was content merely to state that the results became "indefinite" above and below a certain range of concentrations.

POWERS worked on the goldfish Carassius carassius and in solutions of lithium chloride of concentrations ranging from 0.089 to 0.466 he observed that the goldfish died fairly uniformly in any given concentration. When he investigated the toxicity of various concentrations of copper chloride, cadmium chloride and ferric chloride he found that these did not follow the same general pattern as did all the other substances tested. The toxicity of copper chloride at high concentrations (0.66 - 0.25N) increased with a decrease in concentration. The shortest survival time of any fish was 30 minutes. From 0.25N down the toxicity of copper chloride decreased with a decrease in concentration but this was very slight compared with the dilution, until the maximum survival time was 430 minutes. From this work with Carassius POWERS was able to come to several important conclusions. Starting from the established fact that the survival time of the goldfish has a very definite relation to concentration of the solution of the toxic substance and that this relation follows a common general with a very few exceptions, POWERS went on to outline his conclusions which can be summarized as follows:

1. There is a concentration of each of the toxic substances tested which will just cause the death of a goldfish and concentrations below this will not cause death. This concentration POWERS designated as the threshold of toxicity concentration.
2. In concentrations of a toxic substance just above its threshold of toxicity concentration, the velocity of fatality of the goldfish (as measured by the reciprocal of the survival time) is increased very slowly with increasing concentration of the solution of the toxic substance.
3. In stronger concentrations the velocity of fatality is increased more rapidly with the increase in the concentration of the toxic solution.
4. At very high concentrations the increase of velocity of fatality is less rapid in proportion to the increase in concentration of the toxic solution.

Thus the work of POWERS begins to demonstrate that the whole subject of toxicity is not as simple and free from complications as up till that time it would seem to have been imagined. POWERS was, however, dealing with only one animal and although it may be likely that these observations are valid for a wide variety of fish types it may not be possible to apply them directly to aquatic invertebrate phyla.

POWERS explains the changes in the slope of the fatality curve by suggesting that a weak toxic solution tends to accelerate

the general metabolism of the organism, while a very strong one retards it, and that these accelerations have a reflex influence on the rate of the original toxic action. POWERS also defines his "threshold level" in a theoretical sense by saying that the production of the straight line portion of the velocity of fatality curve the "theoretical velocity of fatality curve" is achieved. He describes this as being a straight line which cuts the abscissa at a point designated as the "theoretical threshold of toxicity".

CARPENTER took up this approach using POWER's basic terminology in a study she carried out in 1926 on the lethal actions of soluble metallic salts on fish. CARPENTER used the minnow, Leuciscus phoxinus as her test animal and produced the following curves

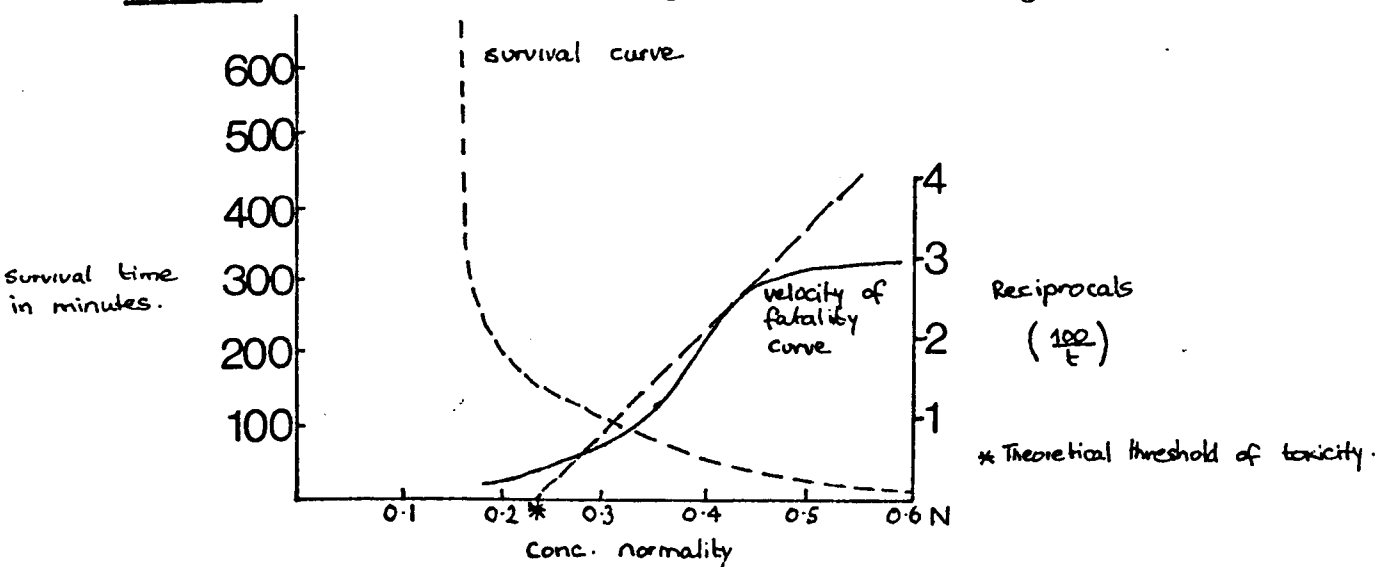


Fig.1. Adapted from toxicity of NaCl to L.phoxinus (CARPENTER,1926)

She explains these curves, theoretically, as follows: the horizontal limit of the survival curve corresponds in general to the equation:

$$y(x-a) = K$$

where: y = survival time

x = concentration

a = theoretical threshold concentration

K = a constant dependent upon the toxic substance used.

Since the actual incidence of fatality stands in inverse relation to the survival time, the reciprocals of the survival times (calculated as $\frac{100}{t}$ to avoid fractions) are plotted to give a curve known as the "velocity of fatality curve". This, CARPENTER says, is very constant in form, being typically sigmoid, i.e. as POWERS had already pointed out and explained, the velocity of fatality increases with concentration most rapidly in the middle portion.

Thus it is seen that the work of POWERS and CARPENTER had by 1926 given the field of heavy metal toxicity a basic theoretical terminology which could be applied to the results of practical toxicity testing procedures. This aspect of the work will now be abandoned in favour of pursuing a survey of the work which has been carried out in an effort to provide some sort of explanation as to the actual physiological causes of toxicity.

Up to this point most of the work described has dealt exclusively with the outward manifestations of the toxicity of certain metals to certain organisms as shown by various experimental and theoretical procedures. In 1920 POWERS had defined toxicity as "the effect resulting from any agent, the presence of which causes the death of the fish or interferes directly in any way with its reproduction, development, growth or normal metabolism". The effect having been defined it is now time to turn our attention to the cause.

In his paper of 1920 from which the above definition is taken, POWERS describes the effects of temperature upon the general "laws" of toxicity which he had propounded in 1917. Although this was not directly an attempt to determine the mechanisms or the nature of the mechanisms that bring about toxicity it does seem to point the way to a broadening of approach towards toxicity problems. Also a study into the effects of temperature would seem to be a good starting point for an investigation into the possible physiological causes of toxicity since physiological processes are very much affected by temperature. In this study, then, POWERS determined the effect of temperature on action of Lithium chloride and Ammonium chloride. He found that the toxicities of both these salts increases with a rise in temperature and that the relative toxic activities of Lithium chloride, at different temperatures, to the goldfish (C. carassius L.) follow very closely the square root of relative standard metabolism of vertebrates as given by Krogh.

An attempt to explain the mechanism of copper + gold toxicity came in 1925 when VOEGTLIN, JOHNSON and DYER published a paper entitled "The protoplasmic action of copper and gold". This paper begins by critising NAEGELI for assuming that the absolute amount of heavy metal involved is so small as to exclude a purely chemical effect and that therefore the toxic action is due to a physical factor of unknown nature. As VOEGTLIN and his co-workers point out, NAEGELI had neglected to take into account the relative mass of protoplasm and of heavy metal salt. They argue that a thread of Spirogyra may weigh only a fraction of a milligram and on exposure

to a large volume (100 cc) of an even very dilute copper sulphate solution (1:10 million) enough metal ions are present to exert a toxic effect by chemical means. The large surface area of such minute organisms as Spirogyra and bacteria furnishes ideal conditions for adsorption of the metal with subsequent storage in the cells. These workers found, in support of this argument, that in a given volume of a toxic solution the toxic effect disappears when the absolute mass of Spirogyra is increased. Experiments which they carried out on two small tadpoles (each weighing approximately 10mg) showed that a copper sulphate solution of concentration 1:1,000,000 killed the tadpoles in $2\frac{1}{2}$ hours, whereas they found that two larger tadpoles weighing 500mg survived in a solution of copper sulphate of concentration 1:500,000 for an indefinite period. They concluded from this that the fact that the larger tadpoles survived is simply due to the larger mass of protoplasm making the toxic threshold concentration more difficult to reach. From this they went on to a consideration of the chemical considerations of protoplasm which could enter into the reaction with these traces of heavy metals and how it is that the cell is injured and killed. The action of higher concentrations of heavy metals, it is argued, may be due to the coagulating action on cellular proteins but in high dilutions it would be difficult to conceive how such small amounts of metal ions combining with a relatively small fraction of the total protein molecules present could bring about a large enough disruption of the physiological systems to cause death. It is also argued that it is more likely that in this case the metal ions exert their action on a

vital constituent which is present only in small amounts. VOEGTLIN and his co-workers settle for glutathione as the possible constituent; this is a dipeptide of glutamic acid and cysteine, it is present in mammalian tissues, yeast and probably also in other organisms. Its function within the cell is concerned with the cellular oxidation/ reduction phenomena. VOEGTLIN et al found that glutathione in its reduced (R.SH) form reacts with copper sulphate and gold chloride with the formation of copper and gold derivatives respectively. Similar compounds were obtained from cysteine. A part of the SH group appears to be oxidized by the metal salt with conversion of the metal ion to a cuprous and aurous state respectively. If these reactions also occur in living cells, then the toxic action of certain heavy metals might be due essentially to a disturbance of the glutathione equilibrium within the cell

$$R.SH \longrightarrow RS + H_2$$

$$R.SH \longleftarrow RS$$

If this were the case death might be seen as due to a special type of asphyxia. If this theory is correct it should be possible to protect protoplasm against the action of metal ions by providing it with an extra supply of reduced glutathione or some other SH compounds. With this possibility in mind VOEGTLIN and his co-workers carried out a further series of experiments in which they made the discovery that albino rats survive an intravenous injection of a minimum lethal dose of sodium cupritartrate if they are also injected with a sufficient amount of glutathione or cysteine. They tried using the other amino-acids, glucose, lecithin and other protoplasmic constituents

but found that these had no effect, i.e. no protective action. Further, they found that the haemolysis and loss of body weight of sub-lethal doses of copper can be prevented by supplying appropriate amounts of glutathione. The toxic action of copper sulphate on Spirogyra was also antagonized under certain conditions by the presence of glutathione but not by an equimolar concentration of the other compounds. What the 'certain conditions' were this paper neglects to disclose.

This experimental evidence, it is claimed by these workers, fully substantiates their hypothesis of the toxic action of copper and gold on protoplasm from widely differing sources. They conclude this study by suggesting that their results should be corroborated by work on other organisms, especially bacteria, before any generalizations should be accepted. Although there is a certain lack of detail concerning both procedure and experimental results which one finds disturbing in this paper, it is nevertheless a serious attempt to explain the phenomenon of heavy metal poisoning. It is a pity that more extensive work along these lines does not appear to have been carried out to any great extent subsequently.

Another paper which sheds some light on the actual biology of heavy metal toxicity is that by MALLORY which was published in 1925. Much of this study deals with cases of haemochromatosis which were thought to have been caused by excessive alcoholism of all things! This study was carried out at the time of prohibition and the alcohol which these unfortunate people procured was, of course, being supplied by 'bootleggers' who were industriously and profitably

engaged in the manufacture of the spirit in copper stills. Upon chemical analysis this liquor was found to contain, among other things, fairly large amounts of copper. Now the symptoms of haemocytosis in the patients examined was pigmentation and necrosis of cells in certain organs but mainly on the liver. These case histories,backed up in many cases by post-mortem reports, led to a series of interesting experiments on rabbits. Five rabbits were given copper powder (100-200 mg each) on their daily food. In two months two of the rabbits died and their dissection revealed pigmentation and the beginning of necrosis of the cells of the liver. The pigment occurred as yellow granular bodies within the liver cells and in the endothelium lining the sinusoids of the liver, the capillaries of the heart, in some of the renal cells and in fibroblasts around the larger blood vessels in the liver and kidneys. Some coarser granules of copper were being passed out in the faeces. In another experiment a rabbit was injected intravenously with 50mg of copper powder in a gelatine suspension and with a 100mg injection the next day. It died in 36 hours. Haemofuscin was the chemical that was identified as being the cause of the pigmentation and granules of this were found already present in the liver cells, especially at the peripheries of the lobules, and to a lesser extent in the fibroblasts around the larger blood vessels. 12mg of copper injected intratracheally into a rabbit caused the necrosis of lung tissue with an acute inflammatory reaction. The liver contained considerable amounts of haemofuscin.

These experiments demonstrated clearly that copper powder

obtaining entrance into the body through the gastrointestinal or respiratory tracts is readily absorbed and causes the deposition of a yellow pigment, haemofuscin, in the liver, heart, kidneys, bone marrow and probably other organs. The same was found to be true of copper powder injected intravenously and subcutaneously. All these experiments were repeated with a suspension of copper powder in distilled water and gave identical results.

It was further found by MALLORY that chronic poisoning with copper in sheep (which are very susceptible to this) and rabbits caused, in addition to the above symptoms, blocking of the tubules of the kidneys with haemoglobin casts, indicating the destruction of red blood corpuscles. MALLORY concludes by suggesting that perhaps all that chronic poisoning with copper entails is a slight but persistent destruction of red blood corpuscles with the setting free of the haemoglobin contained therein. This is then converted to haemosiderin, the haemofuscin being merely a long-lived intermediate product. This idea can be summarised thus:

$$\text{haemoglobin} \xrightarrow{+Cu} \text{haemofuscin} \xrightarrow[\text{pigmentation}]{\text{liver}} \xrightarrow{\text{TIME}} \text{haemosiderin}$$

This work represents another attempt at an explanation of the effects of copper and is doubly interesting in that it is one of the few descriptions of copper poisoning in higher animals and man. Also the explanation which MALLORY offers is completely different from the one advanced by VOEGTLIN and his co-worker in the paper described above.

Another investigation into the physiological effects of copper and other heavy metals followed in 1926. This was carried out by COOK, who in his paper describes experiments performed to determine the effects of copper, silver and mercury on respiration. He used the fungus Aspergillus niger for his experiments. Respiration was measured by an indicator method in which the results are expressed with reference to the rate of carbon dioxide production and not with reference to the actual amounts produced. COOK found that when a solution of copper chloride is run into a respiration chamber containing the organism there was no change in the rate of respiration for a definite period of time which depended on the temperature and concentration of the copper chloride solution. COOK found that the rate of respiration suddenly fell off and carbon dioxide production gradually ceased. This preliminary period where there is no change in the respiration rate has been produced only with copper, iron and tin. COOK called this period the 'latent period' with 'induction period' as an alternative term. Of these two terms COOK expressed a preference for the former, and found that the data obtained from these experiments gave the following curve.

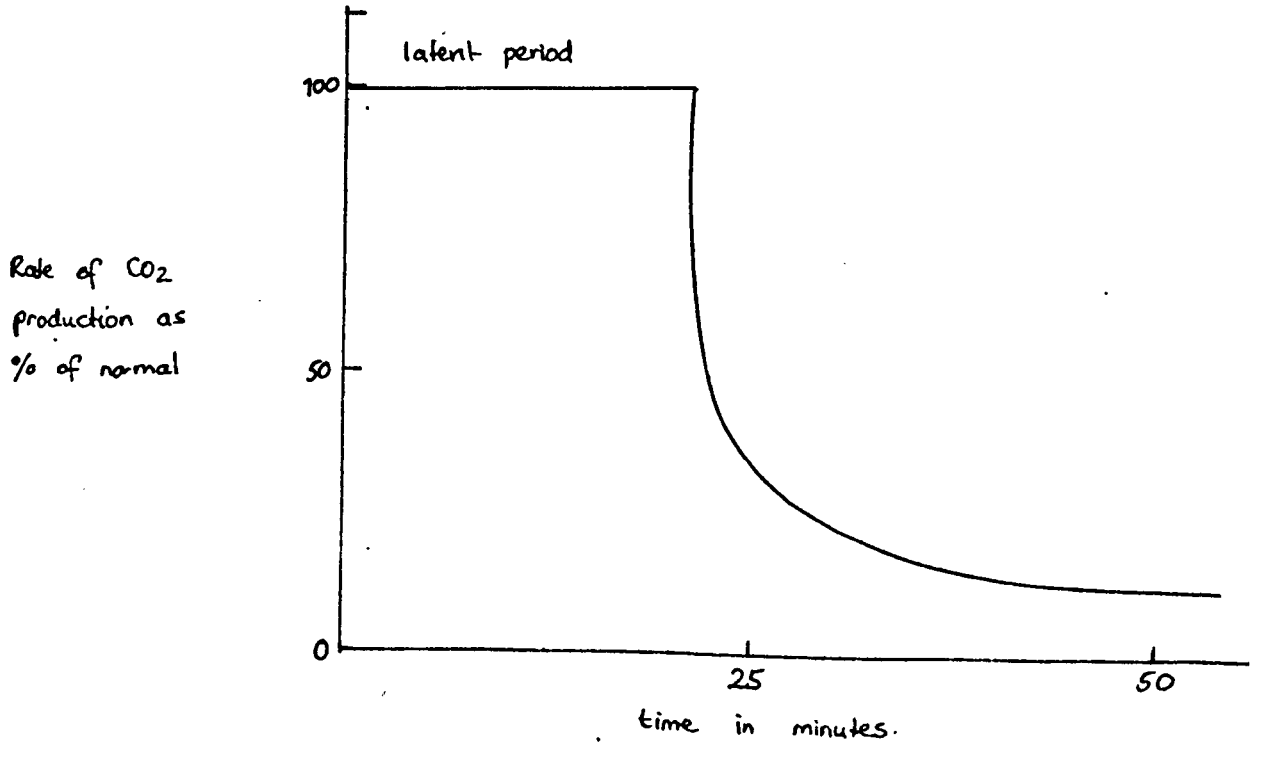


Fig 2 Curve obtained with 0·25 M $CuCl_2$ solution (COOK, 1926).

The interesting thing is that this curve approximately follows the course of a monomolecular reaction curve. In such a curve there is a single substance undergoing decomposition and the amount decomposed in unit time is proportional to the amount present. Therefore, if a is the amount originally present and x is the amount present after time t, then the rate of transformation $\frac{dx}{dt}$ will be:

$$K(a-x)$$

or $$K = \frac{I}{t} \log \frac{a}{a - x}$$

where K is the velocity constant of the reaction.

In the case of a decreasing rate of respiration K could be called the velocity constant of the toxic action.

COOK concludes by putting forward a hypothesis for the action of heavy metals based on the then accepted theory of cell respiration. This states that carbon containing substances are oxidized by the instrumentality of catalysts. Opinion differed as to the nature of these catalysts and their mode of action and COOK had to assume that respiration proceeds in a series of consecutive reactions, each step being catalysed by a different substance or by the same substance in a different state. To explain the mechanism of the toxic action of heavy metals COOK puts forward the idea that these substances alter the velocity constants of certain reactions involved in respiration, either increasing or decreasing them. One point of importance in this theory is that the metals are thought of as either inhibiting or accelerating the reactions governing the formation of the catalysts. Even in the light of present-day knowledge concerning respiration

this theory could be valid but again this line of attack on the problem does not appear to have had any substantial follow-up and where there could have been a comprehensive series of studies in which the effects of heavy metals on the respiration rates of a large and diverse number of organisms could have been studied there has been none.

COOK himself published a second paper in 1926 in which he looked more closely at the latent period which he found associated with the action of copper on respiration rates. In this study COOK starts from the observation that copper differed in its effects from those of other metals studied in that copper chloride has the initial latent period during which the rate of carbon dioxide production is not affected. COOK argues that if this latent period is nothing more than the time required for a chemical reaction to take place somewhere inside the cell wall, then it ought to be possible to demonstrate the presence of copper inside the cell before the end of the latent period. In order to do this COOK suggests two methods of procedure, a) a direct method, testing chemically for the presence of copper in the cell, and b) an indirect method to see if copper can injure the cell within the requisite time using criteria other than that of respiration rate. For these experiments COOK used an unspecified species of the genus Nitella, since A.niger was not large enough for these purposes. Also COOK found that Nitella was a little more sensitive but that the latent period was longer. After determining the respiration curve, cells were placed in the same concentration of copper chloride for varying lengths of time

and the sap then squeezed out. This was tested with potassium ethyl xanthate which gives a yellow to brown with copper depending on the concentration. In several experiments no colour was obtained until 20 to 25 minutes exposure to 0.01 M CuCl. Thereafter COOK found that the colour steadily increased. Rather than concluding from this that copper was not present during the latent period COOK thought it more probable that it was there but in such minute quantities as to be below the threshold for determination with xanthate. COOK also applied his indirect method using Nitella. Separate lots of 25 cells each were exposed for periods of varying duration to 0.01M copper chloride. These were then placed in water and allowed to stand for several hours before testing. A control lot of the same number of cells which were not exposed to copper was placed in water with the other cells. For a criterion of injury COOK chose the turgidity of the cells. If a cell was able to support its own weight when held by one end it was considered to have not been injured. If, on the other hand, it broke down and fell it was considered injured if not dead. A criticism of this technique seems to present itself at this point since it is possible that loss in turgidity could have been brought about by mechanical damage caused by the handling of the cells. However, the results of these experiments showed that even a very short exposure to copper (2 minutes) caused some of the cells to lose turgidity. Of those which had been exposed for longer periods more were injured, until in lots exposed for an hour or even longer all were injured. COOK, therefore, concludes that this shows quite

clearly that the copper injured the cells, in many cases before two minutes had elapsed. In order to injure, the copper must have penetrated at least as far as the plasma membrane and must be exerting its toxic effect on the protoplasm even while it was not affecting the rate of respiration. COOK repeated the above experiments on Valonia with similar results.

The rest of this paper is mainly devoted to the relationship between concentration and the duration of the latent period. COOK found in his experiments with Aspergillus that when the concentration is varied the duration of the latent period varied likewise. This is an interesting relationship because it is the inverse of that observed in the case of velocity of toxic action, i.e. the length of the latent period varies inversely as a constant power of the concentration:

$$\log \text{latent period} = \frac{I}{B} \log C + \log A.$$

This fact, COOK suggests, points to a chemical rather than to a physical explanation of the latent period. Next, in this paper, follows a discussion in which COOK attempts to construct a hypothetical system, on a chemical basis, whereby the process underlying the latent period may be generally illustrated if not fully explained. This hypothesis rests on the assumption that the copper acts on the velocity constants of the participants in the respiratory processes by means of a reversible reaction with a hypothetical constituent within the cell. COOK found that by using appropriate velocity constants the experimental curves could be duplicated by calculated curves.

Another imaginative approach to the problem of heavy metal poisoning was published in 1926 by REZNIKOFF. Here an attempt was made to distinguish between a true, internal physiological effect in sensu strictu of certain substances in living cells, as opposed to a surface effect. Up until that time it had been assumed that the rapidity with which a certain cell of tissue reacts indicated ease of penetration. REZNIKOFF points out that this is not necessarily the case and even where some change has been noted inside the cell one could not be certain that it might not be due to a surface effect which involved a secondary change within the interior or that it was not caused by the abstraction of a substance from the interior of the cell. For this investigation REZNIKOFF used a specially adapted microrgical technique to study the mechanism of the reactions between salts and protoplasm. Using this technique substances could easily be brought into direct contact with definite parts of the cell (REZNIKOFF used both injection and immersion techniques) which in this case was Amoeba proteus.

REZNIKOFF found that when Amoebaewere immersed in copper chloride solution, during the first three days the animals died in a considerable range of concentrations. In solutions weaker than M/4,000,000 toxicity decreased abruptly. Injection with copper chloride caused solidification of the affected region and a disintegration of the adjacent surface. With M/16 copper chloride the Amoebae could pinch off the affected region. Strong solutions solidified the entire cell. Not until a dilution of M/8000 was reached did this solidification process cease. Also, each

successive injection of copper chloride in concentrations ranging from M/16 to M/8000 caused the pinching off of the solidified area with its disintegration surface. After this the remnants appeared to be quite normal except for a temporary and moderate enlargement of the contractile vacuole. This occurred also with very dilute solutions which produced no solidification. From the results of this work REZNIKOFF concluded that the lethal action of the chlorides of lead, mercury, copper, iron and aluminium were due to surface action since the animals did not die when injected with the solutions but only on immersion.

Work such as that described above made it clear that the toxic action of copper and other heavy metals could be due to a variety of causes having their modus operandi in either external, surface reactions or to more truly physiological, internal reactions, or a combination of both. Much seems to depend on the type of organism used for the investigation. In 1927 CARPENTER published a paper describing the lethal action of soluble metallic salts on fish. This investigation sprang from several years work on the fauna of streams polluted by local lead mining and which revealed the fact that the destruction of fish life in such streams was neither a matter of starvation or emigration due to lack of food, or of spawn destruction, but the direct consequence of the action of lead salts in solution (probably the sulphate) upon the fish themselves. Subsequent experiments which CARPENTER carried out both in the field and in the laboratory had shown that lead in the ratios of 1:3,000,000 may be lethal to Minnows, Sticklebacks and Trout.

CARPENTER's paper of 1927 deals with the physiological nature (if any) of this action in order to discover whether it followed the normal course of toxic action as worked out by POWERS. For the most part CARPENTER found that her results agreed moderately well with the 'theoretical values'. Like POWERS she used a wide range of salts ($ZnSO_4$, $CaSO_4$, $FeSO_4$, $FeCl_3$, $CuSO_4$, $CuCl_3$, and $HgCl_2$). The greatest divergence from the 'theoretical values' occurred with copper chloride, ferric chloride and ferrous sulphate and CARPENTER explains that in the first two the irregularity may have been due to the colloidal peculiarities of these salts. In the case of ferrous sulphate she suggests that the discrepancy was probably due to the rapidity with which oxidation takes place in this salt. The general conclusion which CARPENTER draws from this study which is really significant and interesting is that the lethal action of soluble salts of heavy metals on freshwater fish consists of not a true physiological effect but of the formation of an insoluble compound of the metallic ion when it reacts with some constituent of the mucus which is secreted by the fish and thus forms an impenetrable layer over the gills and skin which prevents gaseous exchange. Death is therefore caused by a type of asphyxiation. This action can be lethal even at extreme dilutions, and the economic and commercial importance of this is stressed by CARPENTER since 'it puts an entirely new complexion upon the question of the treatment of mine effluents for the preservation of river fisheries'. The conclusion that death is caused by suffocation resulting from a physical barrier of impregnable mucus was

actually deduced when chemical analyses of residues showed that traces of metallic ions had actually not penetrated into the body of the test fish. Also there was visual evidence since the film could actually be seen on the gills and skin. Where insufficient ion was present this film was shed and complete recovery took place. At first this was observed only in the case of lead salts, but it was later found that other metallic ions also caused death in the same way.

This aspect of toxicity does not seem to have been taken up by other workers until 1945 when WESTFALL had a paper published entitled 'Coagulation film anoxia in fishes'. Here WESTFALL states that due to the absence of specific proof it did not follow that physical changes in the state of the mucus film covering a fish is accompanied by a decrease in permeability to dissolved oxygen. Here, therefore, WESTFALL is questioning what was really only an assumption made by CARPENTER. WESTFALL goes on to describe a series of experiments which he devised to determine the effects of coagulation films on survival during exposures to reduced oxygen concentrations. For these experiments WESTFALL used Goldfish as his test animals and he exposed them to three chemicals in three groups of experiments. The compounds he used were sulphuric acid, lead nitrate and lactic acid. At pH 4.5 he found that after a period of two hours ten fish showed no variation in behaviour in the sulphuric acid, and no coagulation films were noted on the gills of any of the test fish. In a second series a pH of 2.8 was maintained and sulphuric acid at this strength was found to precipitate a substance on the gill mucus and usually death followed within two hours. This experiment was in water

containing only 1 ppm dissolved oxygen. Another group of fish at pH 2.8 but in water containing 5.7 ppm dissolved oxygen lived for an average of 95 minutes. Fish subjected to not only a pH of 2.8 but also to only 1.2 ppm dissolved oxygen had an average survival time of only 53 minutes, i.e. those fish in water containing 5.7 ppm dissolved oxygen lived approximately twice as long as the group subjected to only 1.2 ppm dissolved oxygen.

WESTFALL argues that since the fish in an acid medium with a low dissolved oxygen content in the water died much sooner than those in the same pH but with more dissolved oxygen, it did indeed seem that the permeability of the membrane system of the gills was decreased with the development of the coagulation film. This in turn reduced the intake of dissolved oxygen. This conclusion, however, does not necessarily follow since it is more than possible that the detrimental effects may have been due simply to the low pH value causing physical damage to the delicate gill membrane system of the fish. WESTFALL seems to have thought of this possibility since his next series of experiments seem to be designed to eliminate it. What he did was to repeat the experiment using lead nitrate, a salt capable of precipitating the gill mucus without increasing the acidity of the medium to less than 4.9. Ten fish were placed in a solution of 5g/litre lead nitrate in which the dissolved oxygen concentration was maintained at 6.2 ppm. These fish survived the full two-hour period of the experiment and showed no reactions other than some increased respiratory movements and a slight difficulty in maintaining equilibrium. A second test using ten fish was carried

out in a solution with a dissolved oxygen concentration of only 1.4 ppm. All the fish in this test died before the two hours had elapsed, the average survival time being 93 inutes. It therefore appeared, from this data, that precipitation of gill mucus whether by acid or by a salt of a heavy metal did increase the permeability of the gill in relation to oxygen intake.

In this series of experiments the lactic acid was used to determine the lethal effect of low dissolved oxygen concentrations in an acid medium when there is no apparent precipitation of gill mucus. Ten fish at pH 4.0 with 6.1 ppm dissolved oxygen resulted in no deaths over a two-hour period. A second series of ten fish at the same pH but with only 1.4 ppm dissolved oxygen gave identical results, i.e. no deaths in a two-hour period were caused by merely lowering the concentration of dissolved oxygen in the test water. WESTFALL's work, therefore, would seem to confirm the theory of the lethal effect of certain substances being due to suffocation caused by the reaction of these substances with the mucus of the fish to produce an impermeable film. It is a pity that WESTFALL did not carry out his experiments with more salts of heavy metals. Also an analysis of the residues of certain internal organs might have yielded interesting results.

In the field of heavy metal toxicity a large contribution has been made by JONES, who between the years 1935 and 1947 had published a series of papers dealing with various aspects of this type of toxicity. Much of this literature describes studies made on the toxic action of heavy metal salts on various animals, e.g.

Gastrosteus aculeatus (1935, 1938 and 1939), Polycelis nigra and Gammarus pulex (1936), Bufo bufo bufo (1939) and Pygosteus pugitus (1947). It is not my intention here to describe every toxicity test which JONES carried out since the type of work he has been engaged in can be indicated by reference to only a few of his papers.

One of these which JONES hadpublished in 1936 was called 'The toxicity of dissolved metallic salts to Polycelis nigra (Muller) and Gammarus pulex (L.)'. Here JONES points out that the study of the toxicity of metallic salts to aquatic animals had been mainly confined to members of the vertebrate phyla. He is seen, therefore, to be a worker who has grasped the importance of work with invertebrate aquatic animals. This paper deals with a study of the toxic

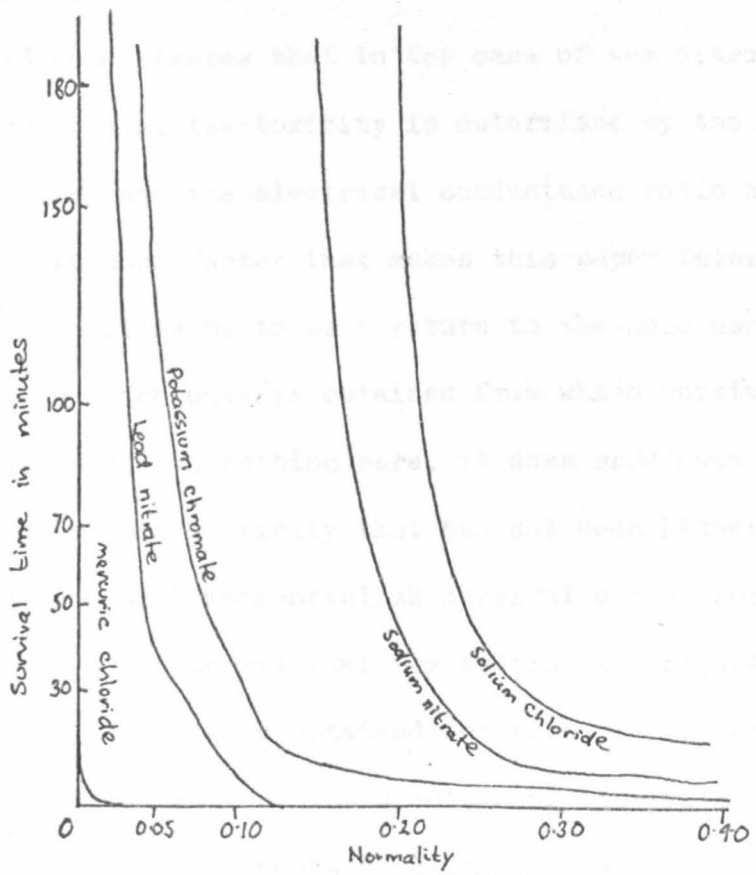


Fig.3 Survival curves for P.nigra in lead nitrate, potassium chromate, mercuric chloride, sodium nitrate and sodium chloride. (JONES, 1936).

action of some variety of metallic salts upon the flatworm Polycelis nigra and the crustacean Gammarus pulex and is mainly a study of the relationship between survival time and concentration. The behaviour of P.nigra in solutions of metallic salts is described and the survival curves for this animal in a variety of salts are shown.

This study is interesting because in it JONES is able to describe a phenomenon which does not appear to have been previously noted. This is that in the case of heavy metal salts the toxic effect at concentrations below isotonicity is due almost entirely to the cation, the toxicity of the anion being very small. At concentrations above isotonicity, however, the anion and the osmotic pressure of the solution act as additional lethal factors. Also, JONES demonstrates that in the case of the nitrates and sulphates of heavy metals, the toxicity is determined by the product of the normality and the electrical conductance ratio at that normality.

Another factor that makes this paper interesting is that although it seems to be a return to the more usual type of toxicity test in which data is obtained from which survival curves can be constructed and nothing more, it does shed some light on two aspects of heavy metal toxicity that had not been hitherto commented on. Also this work does establish survival curves for invertebrates arrived at by normal toxicity testing techniques thus making them comparable to those obtained for fish. Further, JONES discusses the possible mode of toxic action of the salts he tested. Here JONES states that death of P.nigra in a toxic solution is not due to any factor preventing the animal obtaining oxygen from the water but

seems to be due to fixation of the tissues by the heavy metal salt solutions. The more usual fixatives are acids, salts of heavy metals or a mixture of these. These operate by precipitating intracellular proteins in a chemically inert state, the living protoplasm becoming permeated with a reticulum of solid structures. This would seem to coincide with some of the observations made by REZNIKOFF in 1926. JONES makes no reference to this earlier study, however, and one can only assume that he either did not know of it or that he did not, for some reason, consider REZNIKOFF's work valid.

In another study, published in 1938, JONES investigated the relative toxicities of salts of lead, zinc and copper to the stickleback Gastrosteus aculeatus L. This paper also includes a study into the effect of calcium on thetoxicity of lead and zinc salts. JONES chose these particular metallic salts because as he points out, they were the commonly occurring metallic polluting elements at the time. In this study JONES makes an attempt to estimate the relative toxicity of these three metals by the determination of their lethal concentration limits, but he points out that attempts to compare the toxicities of metallic salts by comparing the survival times of fish at one selected concentration are doubtful since at high concentrations one salt may be more rapidly fatal than an equimolar solution of another, while at low concentrations the relation may be reversed. JONES also criticises the use of the goldfish Carassius auratus (L) as a test animal since this is very resistant to the toxic action of heavy metals and therefore results withthis species are not as valuable for general application to pollution problems as results

obtained with species of greater sensitivity. Further, JONES points out that C. auratus is not an indigenous species of freshwater streams in this country. This criticism, although an obvious one, is interesting in that it seems to represent the first serious objection to the use of the goldfish as a test animal since POWERS first chose it for just this purpose in 1917. Also it seems to underline the passing of toxicity problems out of the hands of the pure scientist into those of the applied or more economically orientated researcher. JONES's investigations here have been directed towards the determination of the lethal limits of concentration for lead, copper and zinc to the universally distributed three-spined stickleback, Gastrosteus aculeatus,L. Experiments with copper solutions indicated a toxicity considerably higher than that of lead or zinc, though at high concentrations (0.10 - 0.20 N) the survival times of sticklebacks in copper nitrate, lead nitrate and zinc nitrate were very nearly equal. The high toxicity of copper to certain freshwater animals had already been known for some time and it is evident that pollution by copper salts must have a far more serious effect on freshwater fauna than pollution by lead or zinc. Water quality criteria introduced since this work was done would seem to take this into account in practical terms but it is interesting to look at some of JONES's results. Table I shows JONES's data for the effect of copper nitrate on mature sticklebacks.

Table I The effect of copper nitrate on mature sticklebacks (JONES, 1938).

concs. in g/c.c. water temp. 14-17°C. pH 6.4 - 6.6.

conc.	average survival time
5×10^{-6}	155 mins.
3×10^{-6}	216 mins.
2×10^{-6}	270 mins.
15×10^{-7}	327 mins.
1×10^{-6}	$6\frac{1}{2}$ hours
7×10^{-7}	10 hours
5×10^{-7}	$11\frac{3}{4}$ hours
3×10^{-7}	$21\frac{1}{2}$ hours
2×10^{-7}	$32\frac{1}{2}$ hours
1×10^{-7}	$55\frac{1}{2}$ hours
8×10^{-8}	79 hours
6×10^{-8}	$4\frac{1}{2}$ days
4×10^{-8}	$5\frac{1}{2}$ days
2×10^{-8}	8 days
1×10^{-8}	11 days
average survival time of 32 controls in tap water	$10\frac{1}{2}$ days

As can be seen from this table, not until a concentration of 1×10^{-8} g/cc is reached do the animals survive as long as the controls. One rather worrying question here is why the controls survived only for $10\frac{1}{2}$ days. Since the life span of even the

average stickleback is presumably longer than 10½ days one must assume that other factors have caused or contributed to this relatively short average survival time.

In 1939 Jones had another paper published in which he describes work which looks more closely at a factor already mentioned in his 1936 study. This is the relation between the electrolytic solution pressures of metal and their toxicity. For this investigation JONES once again used Gastrosteus aculeatus L. as his test animal. Many attempts had been made in the past to relate the toxic activity of metals to their atomic weight, valency or other physicochemical properties. Perhaps the most successful study was that made by MATHEWS in 1904. Here MATHEWS showed that the electrolytic solution pressure of a metal is closely related to the degree of toxicity of its salts and that the toxicity of an ion therefore appears to be determined by its affinity for its electrical charges. In his study JONES determined the lethal concentration limits for the hydrogen ion and the ions of eighteen metals. (Here JONES defines lethal concentration limit as the level to which the concentration of the salt in the surrounding water must be reduced before a definite toxic effect disappears.) According to their lethal concentration limits on a mg/litre basis JONES arranged these ions in order of increasing toxicity as follows:

Sr, Ca, Na, Ba, Mg, K, Mn", Co", Cr",Ni", Au", Zn, Cd", Pb",Al, Cu", H, Hg", Ag.

On a molar concentration basis the order was found to be:

Na,Ca,Sr,Mg,Ba,K,Mn",Co",Cr",Ni",H,Zn,Al,Au",Cd",Pb",Cu",Hg",Ag.

All these ions except for the first six (the metals of the alkalis and alkaline earths) bring about the death of fish by precipitating the gill secretions. The alkali and alkaline earth metals appear actually to enter the body and act as true internal poisons. JONES found that on a Mg/litre or molar concentration basis there is a marked relationship between the toxicity of the metals and their solution pressures. The metals of very low solution pressure (e.g. Ag and Cu), i.e. those whose ions are most ready to part with their charges and enter into combination with other ions or compounds, are the most toxic as they precipitate the gill secretions with extreme rapidity. Metals of higher solution pressure (e.g. Zn, Pb and Cd) act in the same way but more slowly. Manganese, which, of the heavy metals, has the highest solution pressure, takes effect very slowly.

Another aspect of toxicity which JONES studied was antagonism. This phenomenon was,again, observed and commented on before and knowledge of its existence can, in fact, be traced back to 1884 when RINGER described the antagonistic effect of calcium and potassium upon the working of the heart. Antagonism can here be briefly described as the reduction in the toxicity of a toxic substance upon the addition of another substance. This other substance can itself be toxic, non-toxic or even insoluble. Antagonism has been observed between a great variety of substances, between salts, between acids and salts, between salts and alcohols, salts and alkaloids and between distilled water and soot, but by far the greater proportion of the work done on antagonism has been that carried out on the antagonism between the salts of the alkali and alkaline earth metals,

especially sodium and calcium. In 1939 JONES had a paper published which dealt with the antagonism between salts of the heavy and alkaline earth metals in their toxic action to the tadpole of the toad Bufo bufo bufo (L). In another study which was also published in 1939 JONES was able to demonstrate that a marked antagonism exists between the heavy metal salts lead nitrate and copper nitrate in their toxic actions on a variety of freshwater animals. In his work with the tadpoles he had exposed them to mixtures of equally toxic solutions of nickel nitrate and strontium nitrate. He found a makred antagonistic action between these salts, the effect increasing with dilution. Also, in this paper he shows that calcium, magnesium and barium also reduce the toxicity of copper solutions. A very interesting feature of this paper is that JONES makes use of three-dimensional perspective drawings of solid models to show effects on survival curves of different concentrations of mixtures of salts.

Another highly illuminating study by JONES which has helped to broaden the approach in toxicity work is one which he had published in 1946 concerning the oxygen consumption of Gastrosteus aculeatus(L) in toxic solutions. Although much work had already been done on the respiratory exchange of entire organisms, isolated organs and tissues there had been little work done on the effect of dissolved toxic substances on fish respiration. POWERS in 1922 made the first comprehensive study of the effect and degree of toxicity of a wide range of toxic substances to goldfish and investigated the effects of variations of the pH of the medium on the physiology of respiration in fish. Apart from one experiment by CARPENTER (1927) in which it was

shown that Gastrosteus immersed in lead nitrate solution evolved carbon dioxide at rather less than 40% of the normal rate, accompanied by an increase in opercular movement, there does not seem to have been another detailed study along these lines.

JONES used solutions of mercury, copper and lead salts and measured the oxygen consumption and opercular movement. Fig.4 shows his results with mercuric chloride.

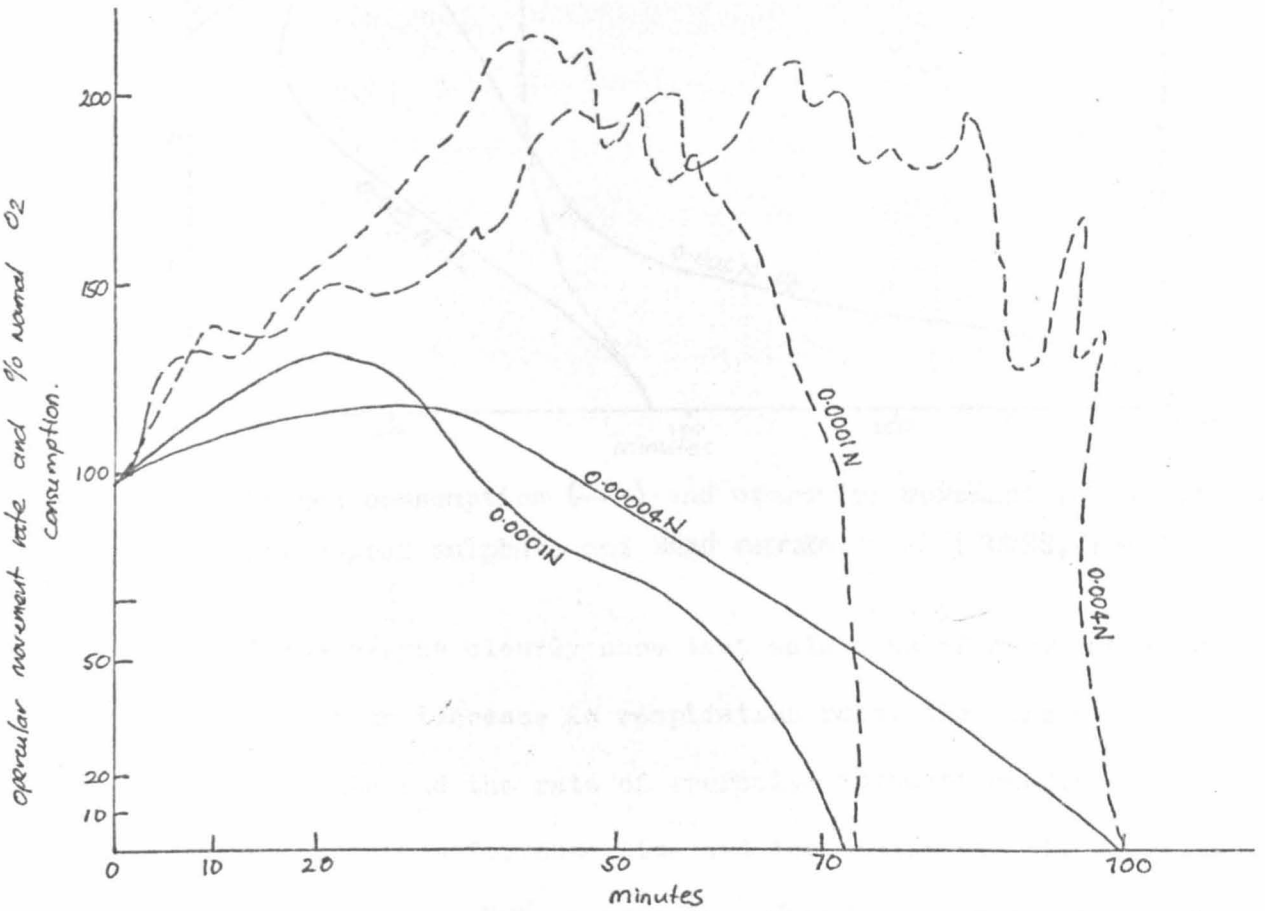


Fig.4 Oxygen consumption (——) and opercular movement (----) curves for mercuric chloride (JONES, 1946).

His results with copper sulphate and lead nitrate are shown in Fig.5.

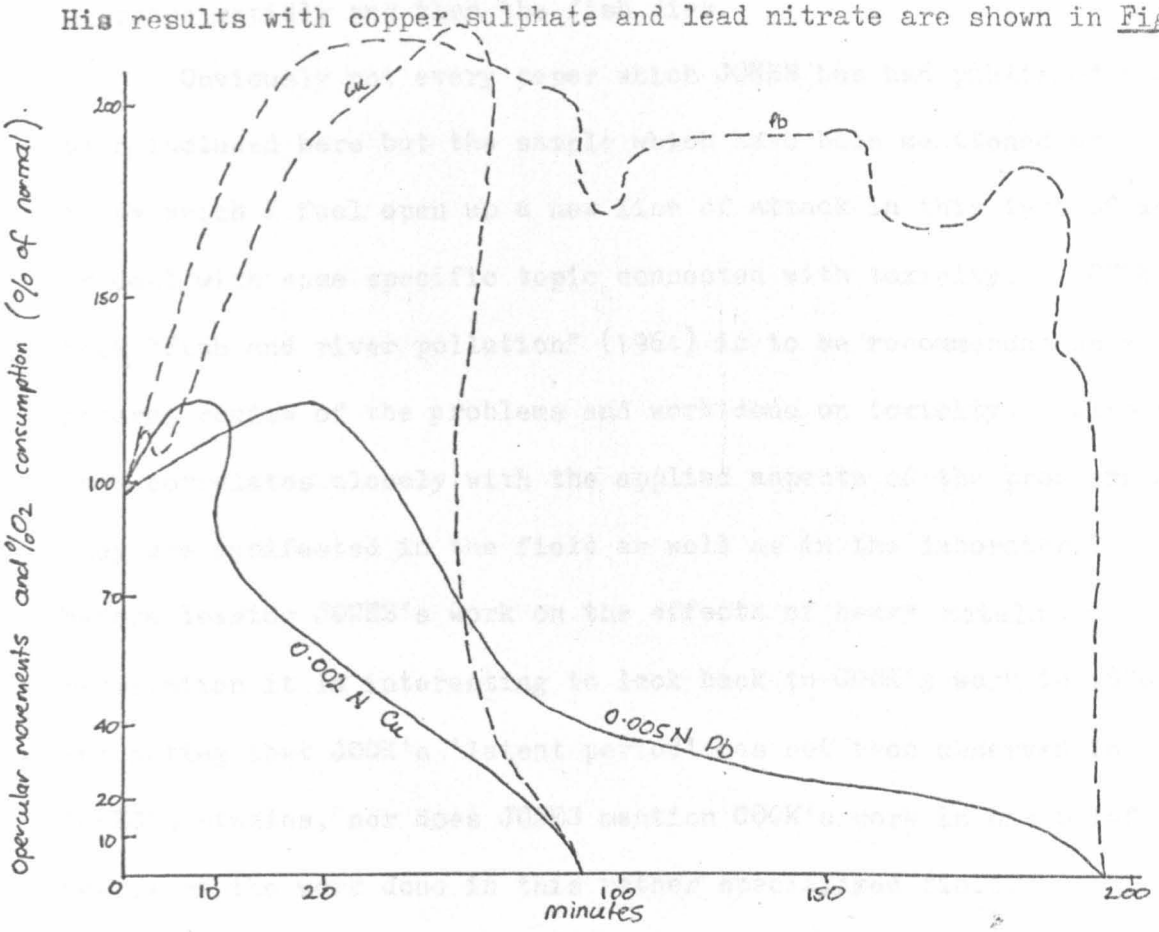


Fig.5 Oxygen consumption (——) and opercular movement (----) curves for copper sulphate and lead nitrate (JONES, 1946).

These graphs clearly show that solutions of heavy metal salts produce first an increase in respiration rate, then the oxygen intake declines and the rate of opercular movement continues to increase, continues for some time and then falls rapidly when the oxygen is reduced to 38% of normal. JONES concludes that in the case of heavy metal salts respiration is obstructed at the gill surface and the carbon dioxide content of the blood is raised so that the respiratory centre is stimulated and opercular movements increase in speed and amplitude. These movements can be maintained only until the oxygen level drops to below 38% of normal when they

decrease rapidly and then the fish dies.

Obviously not every paper which JONES has had published has been included here but the sample which have been mentioned are those which I feel open up a new line of attack in this type of work or deal with some specific topic connected with toxicity. JONES's book "Fish and river pollution" (1964) is to be recommended as a good general review of the problems and work done on toxicity. Also the book correlates closely with the applied aspects of the problems as they are manifested in the field as well as in the laboratory. Before leaving JONES's work on the effects of heavy metals on respiration it is interesting to look back to COOK's work in 1926 and noting that COOK's 'latent period' has not been observed in JONES's studies, nor does JONES mention COOK's work in his brief review of the work done in this rather specialized field.

Soon after this work by JONES a number of papers appeared which described work which had been carried out on marine invertebrates. One of these, published in 1947 by PYEFINCH and MOTT, describes work investigating the sensitivity of barnacles and their larvae to copper and mercury. They used two species of barnacle, Balanus balanoides and Balanus crenatus. This investigation yielded interesting results in that it showed up contrasting degrees of sensitivity in different stages in the life histories of these animals to copper and mercury. The stages PYEFINCH and MOTT studied were the nauplii, cyprids, metamorphosing cyprids and adults. These they subjected to exposure periods of six hours. The results they obtained showed there was a sharp decrease in sensitivity both in

B.balanoides and B.crenatus between the last naupliar stage and the cyprid. Also, they found that settlement of B.balanoides could be prevented by very low concentrations of copper and mercury, even though there were no obvious lethal effects. On the other hand, metamorphosis of the cyprids of both species was not prevented by concentrations of copper as high as 7 mg/l, in sea water, i.e. a sharp change in sensitivity had occurred.

The young barnacles of both species, immediately after metamorphosis, were found to be much more sensitive to copper than the metamorphosing cyprid and copper and mercury appeared to be roughly equi-toxic to the adult of B.balanoides; the adult of B.crenatus was found to be more sensitive to copper and distinctly less sensitive to mercury than B.balanoides.

In the same year as the paper just described BARNES and STAINBURY made an investigation into the toxic action of copper and mercury salts, both separately and when mixed, on the Harpacticid Copepod Nitocia Spinipes (Boeck). This study, the investigation of PYEFINCH and MOTT and several other studies carried out about this time, was carried out in connection with the fact that at the time both copper and mercury salts were being used in antifouling compositions and an obvious need for more information (after the event) as to their effects on animals which were unfortunate to inhabit the environment into which these poisons were being introduced, was being felt. In the course of their investigations BARNES and STAINBURY uncovered two very interesting facts about copper and mercury in the marine environment. Firstly they found a

marked difference in the degree of toxicity of these two metals (this contrasts somewhat with the findings of PYEFINCH and MOTT), and secondly that a marked synergism exists between these two metals.

The median lethal dose for mercuric chloride was found to be 0.6mg mercury per litre. In contrast with this they found that copper sulphate did not kill 50% of the animals at a concentration of 26mg copper per litre! These results, they suggest, point to the conclusion that the mode of action of the two salts may be entirely different. It is possible, however, that the slow increase in effective action with increasing copper concentration (from 0.26 to 2.6mg copper per litre there was an increase of only 11 to 21% in the kill) may be due to the formation of colloidal basic compounds, whose poisonous action may be entirely different from the cupric or cupri-complex ion. Also, it is possible that the behavious of copper solutions could vary with salinity due to the complex solubility relationships in sea water.

Mixtures of copper and mercury were found to be far more effective than either alone and at the same concentrations, and, moreover, the toxicity was frequently far greater than would have been expected on an additive basis. BARNES and STANBURY point out that this striking synergism strengthens the hypothesis that the two metals behave differently towards the animal and that different systems or at least different parts of the same system could be affected. The simplest explanation of this synergy, they suggest, would appear to be that lowered vitality, due to one type of poisoning, does not allow the animal to deal as effectively with the

second poison. Thus, it is suggested, impairment of the excretory system by mercury may allow sufficient accumulation of copper to interfere with the respiratory system. It is worth pointing out that by this time (1947) many workers believed that the main site of attack by copper was the respiratory system. An alternative to this hypothesis which BARNES and STANBURY put forward is that the effect of one poison could change the permeability characteristics of the animal or cell membranes and so facilitate the entry of the second poison. This does not seem to be exactly an alternative to the first theory, since this suggestion is concerned merely with the entry of the poisons into the body of the animal while the first idea is concerned with the internal action of the poisons. These seem to be two separate points and by no means need be mutually exclusive, i.e. both could be happening at the same time.

This paper is concluded by putting forward the possibility that the effect is one external to the animal and not concerned with a true poisoning process. Heavy metals, especially copper and mercury, are readily adsorbed onto colloidal material and in mixed solutions preferential adsorption of one could allow a greater concentration of the second to be available for the poisoning action. This last theory, however, would not seem to offer as convincing an explanation of the marked synergism that exists between copper and mercury as do the first two.

This study would appear to be the first to describe such synergism and clearly it would have been desirable to have had more experimental work carried out into the action of other metals and

synergism. Perhaps a correlation with JONES's arrangement of the metals according to their electrolytic solution pressures and synergism could have been attempted. This avenue of research seems, however, to have been somewhat neglected by subsequent workers.

BARNES and STA NBURY's paper is worthy of note not only because it describes certain phenomena which until then seem to have escaped detection but also because they make some attempt at arriving at an explanation in physico-chemical terms for externally visible phenomena which they detected by means of the toxicity test, i.e. here the toxicity test is being used as a point of departure and not as an end in itself.

Another study made along similar lines to the last two described was that made by CLARKE in 1947 on the poisoning and recovery of barnacles and mussels. Again, this study has its roots in the use of heavy metals as antifouling agents. CLARKE points out that it had previously been found the efficiency of these anti-fouling paints in preventing attachment of organisms to ships' hulls is related to the solution of toxic materials, such as copper, from the paint. Experiments are described which CLARKE carried out to determine the effectiveness of various metallic salts in preventing fouling by barnacles and mussels. The salts tested were mercuric chloride, silver sulphate, zinc nitrate, basic cupric carbonate, cupric citrate, cupric tartrate, cupric salicylate and cupric-p-aminobenzoate. The species of barnacle CLARKE used were Balanus balanoides and Balanus eburneus. He found that basic cupric carbonate was the most toxic form of copper tested but the other copper salts were only slightly less toxic. The toxicity of silver

was slightly less than that of copper but greater than that of mercury. Zinc was found to be very much less toxic than the other metals tested. This series, Zn, Hg, Ag and Cu arranged in increasing toxicity does not, unfortunately, correspond with the arrangement of these metals in JONES's study of electrolytic solution pressures either on a mg/litre basis:

Zn Cu Hg Ag

or on a molar basis:

Zn Cu Hg Ag.

It must be pointed out, however, that JONES was working in a freshwater medium whereas CLARKE's study was carried out in a saline medium. This may account for the discrepancy.

In further experiments with cupric citrate it was found that 2 to 5 days were required to kill both species of Balanus in solutions containing 0.22mg/l, and in concentrations of 0.06mg/l or less the animals remained alive for two weeks or more. CLARKE found that nauplii were killed after much shorter periods of exposure than were the adults. This agrees nicely with PYEFINCH and MOTT who found that the adults were more resistant except immediately after metamorphosis. As to the mussels, CLARKE found that Mytilus edulis was killed in 12 hours by solutions containing 0.55 mg/l of copper. When barnacles which had been poisoned to the extent that they remained open and were inactive but would still respond to touch were placed in fresh sea water, they usually recovered completely within a few days. Mussels which had reached a similar condition never recovered in fresh sea water.

CLARKE analysed the tissues of the barnacles and mussels for copper before and after they had been exposed to solutions of cupric citrate. The concentration of copper in the soft tissues of normal animals was found to be considerably greater than in an equal volume or weight of sea water. After exposure to solutions of copper citrate the concentration of copper in the tissues increased considerably. A certain amount only of this copper appears to have been eliminated when barnacles were returned to fresh sea water. Mussels, on the other hand, were able to eliminate a greater amount of the copper they had taken up, and in some cases after two weeks in sea water the concentration of copper in their tissues had returned to normal levels. It was found that solutions of cupric citrate in the concentrations tested did not prevent attachment of cyprids of Balanus balanoides and very high concentrations of copper (over 116mg Cu/l), were required to prevent metamorphosis of freshly attached cyprids. Moderate concentrations of copper, however, retarded the development of newly-metamorphosed barnacles and prevented the formation of the cemented calcareous base. CLARKE concludes his paper by stating that the effectiveness of antifouling paints containing copper is due to interference in this stage of the animal's life history.

These three papers illustrate the type of work being done as a direct result of the need to know more about the effects that substances which were being introduced into an ecological system were having on the inhabitants of that system. As always, it seems extraordinary that these types of investigation should be initiated after the substance in question has already been introduced into

natural systems. However, interesting results have been obtained in the course of such studies and much information concerning the factors that govern toxicity has been obtained which is valid in itself quite apart from any use which the scientist/technologist may wish to put it to. It is a pity that at the time a more comprehensive study, taking in a much wider variety of marine life, was not undertaken; presumably once the applied aspects of the problems have been looked into interest or financial backing, or both, waned.

Returning to freshwater, a study made in 1948 by PODUBSKY and STEDRONSKY on the toxic effect of several metals to fish and river crabs found that copper was the most toxic, followed by zinc, brass, iron and nickel. Aluminium was found to be toxic in waters containing little calcium and copper coated with zinc, and tin and iron coated with zinc were found to be only slightly toxic. Stainless steel, iron coated with nickel and chrome and lead were found to be non-toxic. In 1950 these workers carried out another investigation on the toxic effects of some metals on both small and large organisms (unfortunately the abstract from which this information was obtained neglects to specify what organisms were used). The effects of copper, zinc and other metals on aquatic organisms is discussed and copper and zinc were found to be markedly toxic to ephemerid larvae but chironomid larvae were found to be fairly resistant.

In June 1951 a note of some interest appeared in the journal 'Nature', by MACKERETHand SMYLY. These two workers reported that in June 1951 Stone loach Nemacheilus barbatulus L., which had been kept alive in an aquarium since March 1951, died when it became necessary

to replace the water supply which came from a nearby reservoir with lake water from Windermere. Chemical analysis showed that the original water contained 0.15 ppm copper (Cu^{++}), whereas the lake water had 0.28 ppm copper. In the laboratory stone loach when placed in water containing copper, in solution, in concentrations from nil to 0.3ppm (in steps of 0.05) lived only in concentrations of less than 0.2ppm copper. At this concentration all but one fish died within 24 hours. MACKERETH and SMYLY therefore concluded that it was reasonably certain that the original fish had died of copper poisoning.

This note is interesting in that it furnishes us with another threshold concentration for another species, also it seems that by pure accident it was discovered that there was a lethal concentration of copper in lake Windermere. Since copper does not usually occur naturally in this typeof concentration it seems reasonably certain that the copper was, either by accident or by design, introduced by man. As can be seen, by this time a whole mass of information and data had been accumulated from investigations which had been carried out into various aspects of heavy metal poisoning. Heavy metal toxicity to aquatic organisms had now become a distinct discipline and testing techniques and methods of processing the data obtained from testing programmes had been evolved and developed to an at least limited degree of standardization. In 1953 DOUDOUROFF and KATZ had published a critical review of the literature on the toxicity of industrial wastes on fish which included a section on the metals. Here the authors review the literature on the toxicity

to fish of simple inorganic salts of metals including studies made on the relative toxicities of various metals, antagonism and synergism of metallic cations, and hydrolysis and precipitation of metal compounds in natural waters, mode of action of metal salts and the role played by anions and the effect, in toxicity tests of temperature, volume of the test solution and acclimatization of fish. Certain metals aredealt with individually and these include aluminium, arsenic, barium, cadmium, calcium, chromium cobalt, copper, iron, lead, lithium, magnesium, mercury, nickel, potassium, silver, sodium strontium, tin and zinc. DOUDOUROFF and KATZ concluded that in dilute physiologically unbalanced solutions of single metal salts all metals are toxic to fish, but in mixed salt solutions the metals are often less toxic because of the antagonism which exists between different metals. They point out, however, that some highly toxic metals such as zinc and copper are strongly synergistic and a mixture of salts of these metals can be much more toxic to fish than are solutions of the individual salts. Also, the toxicity of many metals is markedly affected by temperature, concentration of dissolved oxygen and the volume of the experimental solution. The least toxic metals, it is stated in this review, are sodium, calcium, strontium, magnesium, potassium, lithium barium and manganous and cobaltous ions. Highly toxic metals were found to include silver, mercury, copper, lead, cadmium, aluminium, zinc, nickel and trivalent chromium. Hexavalent chromium, in solution as chromates and dichromates, is less toxic than the trivalent form, in simple solutions of chromic salts. Solutions of permanganates are

much more toxic than solutions of manganous salts containing an equivalent concentration of manganese. This resume of most of the information available at the time concludes with the observation that the toxic concentrations of iron had not been established accurately and that further work on this was required.

This review represents the sum total of knowledge on the toxicity of heavy metals which had accumulated since serious work in this field had begun in 1894. As can be seen, much has been established on a wide range of metals concerning their individual and joint toxicities on certain test animals. This formed the basic pool of information from which more sophisticated work could be done and more searching questions, hopefully, could be answered.

In 1954 a highly interesting paper was added to the literature. It was by SHAW and presented a highly idealized, theoretical model of cation toxicity. Once explained, the model was confronted with actual experimental data from the literature; it had as its starting point the assumption that cations are toxic because they combine with an essential sulphhydryl group on a key enzyme. A second assumption is that the affinity of the cations for the sulphhydryl group is a linear function of (or at least directly proportional to) the insolubility of the corresponding metal sulphide. This would indicate that the metal ions that form the most insoluble sulphides will be the most toxic. SHAW goes into highly theoretical and mathematical considerations based on the inhibited Michaelis-Menton mechanism and the principle of linear free-energy changes. This leads to the prediction that the negative logarithm of the metal ion concentration just necessary to cause the death of an average specific organism (pI^*) is related to

the negative logarithm of the solubility product constant of the corresponding metal sulphide(pKsp) by the following equation:

$$pI^* = S + (m)\ pKsp.$$

The parameter S is the susceptibility of the organism to the poison and would be expected to differ from organism to organism. The parameter m is a proportionality constant and should be entirely independent of the organism studied. A plot of pI^* against pKsp for various organisms should result in a family of straight lines with different intercepts but a common slope m. SHAW applied this analysis to available data and found that his conclusions were justified to a certain extent.

In 1956 SHAW, in collaboration with GRUSHKIN, had another paper published in which he described a follow up to this approach. This paper describes work which was undertaken in connection with three separate points. These were:

a) to obtain additional experimental data on the toxicity of cations to aquatic organisms.

b) to subject all available data to a statistical treatment.

c) to collect other pertinent material from the literature.

SHAW and GRUSHKIN carried out toxicity tests with two different animals, guppies, Lebistes reticulatus and tadpoles of the toad Bufo vallicens. The findings of these experiments plus other data obtained from the literature on Paramecium, Polycelis nigra, Fundalus eggs, the stickleback and Daphnia magna were analysed in terms of the model based on the inhibited Michaelis Menton mechanism, and on the assumption that the cations considered are

toxic because they combine with an essential sylphydryl group attached to a key enzyme.

With the exception of Paramecium it was found that the predictions made with reference to the theoretically derived formula tallied remarkably well with the observed results. This is particularly significant when one considers that several phyla were used in this work. Viewed in this light these results do seem to point strongly to a truly basic mechanism of toxicity. This work is also interesting in that it is, like JONES's work on the electrolytic solution pressures, an attempt to explain one aspect of toxicity in physicochemical terms, and seems to be, also, the first time that a model has been built up on theoretical considerations and actually used to predict experimental results. The idea that there is an essential sulphhydryl group with a high affinity for certain cations has some support derived from the investigations of other workers, especially those of KLOTZ (1952 and 1954) who pointed out that the affinities of metal ions for the sulphydryl group in serum albumin closely parallel the corresponding pKsp values. Also, ALBERT (1952) has observed that there is a high affinity in cysteine for metal ions, and other results indicate the tendency of metals to combine with the -SH group roughly parallels the order of sulphide insolubility. As SHAW and GRUSHKIN point out, however, to postulate that the sulphydryl group is affixed to an enzyme is more difficult to justify although it is plausible. Also there are, in this work, many simplifications and until there has been definite experimental vindication for some of these this work must remain,

for the most part, in the realm of theory.

Returning to the type of study which forms the bulk of work in this field, there is a paper which LLOYD had published in 1960. This deals with the toxicity of zinc sulphate to the rainbow trout. This study is mentioned here because it involves an investigation into the effects of certain physical and chemical variables to the toxic action. LLOYD found that zinc sulphate was less toxic in hard than in soft water and that solutions of zinc sulphate containing calcium chloride were less toxic than those containing an equivalent concentration of calcium as bicarbonate. LLOYD also investigated the effect of temperature and found that an increase in temperature decreased the survival time in zinc sulphate solutions made up in a hard water, but that the threshold concentration was not appreciably affected by changes in temperature. Also, LLOYD found that a reduction in dissolved oxygen concentration of the water increased the toxicity of zinc sulphate, but he also noted that this effect was reduced when the fish were previously acclimatized to the lower oxygen concentration of the test. LLOYD concludes with the interesting observation that the cause of death of fish in the zinc sulphate solutions was <u>not</u> by the precipitation of mucus on the gills but probably by damage to the gill epithelium. Here LLOYD used the radioactive isotope of zinc, ^{65}Zn, to trace the distribution within the body of the fish of zinc taken up from the solutions. The data which LLOYD obtained from this work is shown below. As can be seen, the greatest concentration of zinc found in the tissues was in the gills. LLOYD points out, however, that the

proportions of zinc found here were only 17 and 35% of the total amounts found in the two fish. Also, a considerable proportion of zinc had been distributed throughout the remainder of the body tissues.

Table 2 The distribution of zinc taken up by two rainbow trout killed in a solution of zinc sulphate containing ^{65}Zn as a tracer (LLOYD, 1960).

Sample	g.zinc per g.wet weight tissue	
	Fish 1	Fish 2
whole fish	7.35	11.6
gills	63.2	59.6
gut	0.9	1.7
liver	2.1	1.7
precipitated mucus from post anal region	-	128.0
post anal region after removal of mucus	-	13.8
remainder	5.4	7.25

LLOYD concludes that as there was a considerable proportion of zinc present in the gills of the fish, which was not attributable to a precipitated zinc-mucus-protein complex, it was suspected that the deaths might have been caused by damage to the tissues of the gills themselves. To try to obtain some data to back up this idea LLOYD performed a series of experiments in which the gills of the rainbow trout were exposed to a range of concentrations of zinc and then subjected to histological examination. In sublethal concentrations

of the metal (3ppm.Zn) no deviations from the normal structure could be observed within two days. In moderately high concentrations (4.0 - 4.5ppm.Zn) some swelling of the gill lamellae appeared before death. In very high concentrations (20ppm.Zn) cytological breakdown of the epithelium occurred within 2½ hours. In all cases no excessive secretion of mucus was observed.

As far as I know this, together with an earlier study by SCHWEIGER (1957) in which similar results were obtained, is the only investigation carried out in which an alternative to the coagulation film anoxia is presented. It is a pity that work along these lines has not been carried out on a much wider basis, taking in many more heavy metal salts and a wider range of fish. SAIKI and MORI (1955) carried out work with radioactive zinc on a variety of fish, but these workers found the greatest concentration of zinc found in the body after 45 hours to be in kidneys with only a small proportion in the gills. Such conflicting results make this part of the story far from finished.

LLOYD's work, together with that of SCHWEIGER, does seem to show fairly conclusively that the gills of fish killed in solutions of heavy metals (SCHWEIGER used cadmium and manganese) are, in certain circumstances, severely damaged. This would seem to suggest that the heavy metals may not be general internal poisons but that they act specifically on the gills of fish. LLOYD concludes by observing that the precise action of the heavy metals in causing such damage to the gills is not known. This would seem a worthwhile line to follow up in future work.

Other papers by LLOYD include a study into the toxicity of mixtures of zinc and copper to the rainbow trout (1961) and a review of the work done on the effects of physical and chemical factors on toxicity in general (1962). LLOYD's work seems to have followed a fairly conventional pattern of procedure in that the toxicity test constitutes his main weapon of attack. Other studies, with radioactive isotopes, seem to have come about in an incidental way and seem to be considered as secondary aims, the primary aims being concerned with the establishing of lethal concentrations. LLOYD has, however, been responsible for new work and the bringing together of previous work on the effects of physical and chemical factors on heavy metal poisoning.

A very interesting paper in which work with radioactive isotopes is given real prominence is that by YAGER and HARRY. This appeared in 1964 and is concerned with the uptake of radioactive zinc, cadmium and copper by the freshwater snail, Taphius glabratus. In a previous study (HARRY and ALDRICH, 1963) it was found that zinc, cadmium and copper were among the more toxic of 22 ions tested on the T.glabratus. At concentrations of 0.050 to 0.10ppm cadmium and copper produced a condition termed distress, in which the snail was extended but unable to attach the foot or crawl. This later work of YAGER and HARRY was undertaken to gain additional information on the effect of these three heavy metal ions in relation to the normal and distressed states of the snail, by using radioactive isotopes.

Laboratory-reared snails were exposed to concentrations of

zinc 65, cadmium 115M and copper 64 which allowed normal behaviour, and to concentrations which resulted in the snails showing the distress reaction described by HARRY and ALDRICH. This more recent work showed that much variation existed in the uptake of metal ions by the snails exposed in each experiment. This is, however, thought to be due to some intrinsic property in the behavious of the snails, possibly depending on the activity of each snail while in the test solution. Pairs of snails showing counts of the intact body closest to the average were used for further analysis of the uptake of various parts of the body. It was found that the uptake patterns were essentially the same for all three metals. In normal snails, more metal ions were taken up by snails exposed to large volumes of dosing solutions than was taken up by snails exposed to only 10ml of solutions of the same concentration for equal periods of time. The uptake by the shell decreased in tests in which snails were exposed to 10ml of dosing solution per snail with increasing exposure times. Also, in a given experiment, it was found that the uptake was of the same order of magnitude for all the body parts except the liver, which showed 4-7 times the amounts present in other tissues. The liver in distressed snails had only twice the amount of metal found in other tissues, and snails which showed normal activity often had much larger amounts of metal present in the tissues than did distressed animals. YAGER and HARRY conclude that distress is dependent on the concentration of an ion, rather than the amount of an ion absorbed, and that the ions are effective in producing distress by disrupting membrane

permeability rather than by interfering with some internal metabolic mechanism.

This work is significant since it shows what sort of attempts at gaining information on toxicity could be made when the advancement in radioactive tracer methodology had reached a sophisticated enough level to make such studies possible. Also, this study makes an attempt to trace specifically where exactly these ions are being concentrated within the body. The fact that the liver contained more than other organs is of interest since it would seem to link this work to that of MALLORY's paper of 1925. Also, in this paper there is an attempt to try to explain the mechanism by which these metal ions act which would seem to be a very desirable thing to do after the basic groundwork of lethal concentration etc. has been covered. Again, it is a great pity that this line of approach has not been taken up with a much wider range of animals since only then can a truly comprehensive picture of not only lethal concentration limits, but also of the possible physiological reasons behind them, be built up.

In contrast to this paper is a study published about the same time (1964) by HERBERT and WAKEFORD entitled 'The susceptibility of salmonid fish to poisons under estuarine conditions. I zinc sulphate'. Here the resistance of yearling rainbow trout and Atlantic salmon smolts to zinc sulphate is investigated. This was found to increase with salinity up to 30-40% sea water, in which these species can withstand for two days, respectively, 15 and 13 times as much zinc sulphate as in freshwater. It was found that further increase in salinity to 72% sea water reduced the tolerance

for the zinc salt. Also salmon smolts were more susceptible to zinc poisoning than trout in fresh water and at all the salinities tested.

These last two papers, both published in 1964, show clearly two different approaches which were being employed. The former is primarily concerned with the determination of the physicochemical principles, whatever they are and wherever they are, that lie behind heavy metal poisoning, while the latter is concerned with the 'toxicity test' and not very much else. It is desirable and even necessary that this approach is used before going on to deeper probings but it seems that it has often been the case where studies involving the determination of lethal concentrations, tolerance limits etc. for a particular species have not been followed up with work which could give information as to the mode of action of a particular heavy metal.

Another example of a study of the 'toxicity test' type is that by MOUNT which was published in 1967. This deals with the chronic toxicity of copper to fathead minnows (Pimephales promelas, Rafinesque). Here the fish were exposed to copper sulphate for eleven months to find the fraction or percentage of the median tolerance limit that does not affect growth and reproduction under prolonged, continuous exposure. The results indicated that the true value lies between approximate limits of 3 and 7% of the 96-hour Tlm. MOUNT points out that the absolute copper concentration that is toxic will depend on certain characteristics of the water but may be estimated using the above percentages of the acutely toxic concentration as determined in the water concerned.

Another paper which seems to stress the value of having a prolonged exposure time was published in the same year by HUBSCHMAN on the effects of copper on the crayfish Orconectes rusticus (Girard). This is a valuable addition to the literature since little is known of the effects of copper on theCrustacea. Like MOUNT's work this study is firmly rooted in the applied aspects of the problems and it determines accurately the effect of antifouling compounds on the life that inhabits the ecological system into which they are introduced. Also, HUBSCHMAN makes some significant theoretical points in this study. He draws attention to the fact that there is little known concerning the physiological effects of heavy metals in small concentrations over long periods of time. Also, he points out that the dividing line between acute and chronic toxicity is difficult to define and that to study the mechanisms of chronic toxicity it was first necessary to determine the levels of copper that were acutely toxic under certain exposure conditions. HUBSCHMAN, therefore, carried out a series of preliminary experiments to determine this threshold concentration. These were carried out as continuous flow bioassays, and early in his investigation HUBSCHMAN found that, on the basis of 24 to 96-hour mortality data, adult crayfish appeared to be highly resistant to small concentrations of copper. He later performed experiments to determine any possible delayed effect of short and periodic exposures as well as of continuous exposure during different stages of the life-cycle. This is significant since it seems to be the first time that a worker has grasped the idea that short-term laboratory tests may not be all that meaningful in relation to the

actual environment, since here an organism may be exposed to a substance for a period of time that is very prolonged. It is not unlikely, therefore, that there may be effects which, due to either the resistance of the animal, the concentration of the substance or accumulative factors, may not manifest themselves until after a fairly long period of exposure. Also, a relatively short period of exposure may cause delayed effects which appear only at some time after the original exposure period. The purpose of HUBSCHMAN's study was, therefore, to determine basic environmental requirements for aquatic invertebrates and, as he points out, in terms of water quality criteria it is of little satisfaction to state that an organism survived 24 or 48 hours in a given concentration of a certain substance.

This study has shown that mortality may, in fact, result long after the initial exposure took place. This delayed effect makes it difficult or impossible to define the exact levels of acute toxicity. This one aspect of the problem would seem to throw almost the entire literature up to this point into a whole new light of critical appreciation.

HUBSCHMAN found that as in the case of many poisons, the toxic action of copper is cumulative at certain concentrations. Following 24 hours' exposure to 2.5mg/l, adult crayfish died at a rapid rate (all were dead by the end of 15 days). Animals exposed to the same concentration on an interrupted basis exhibited strikingly similar sensitivity. This study has shown that a concentration of 3mg/l is sufficient to kill 50% of the adult

crayfish exposed for 96 hours. HUBSCHMAN points out that if this value was taken as a guide, severe damage could result, even if the calculated amount was delivered over several weeks. Reduction of concentration by a factor of 10 could still result in destruction of the young population and the following year's brood stock. The application of copper in such conditions, therefore, HUBSCHMAN concludes, is of more than experimental interest. At a concentration of 1 mg/l (the recommended limit for drinking water laid down by several authorities), 50% mortality among newly hatched young was reached with an exposure time which was 1/50th of that required for adults. This, HUBSCHMAN points out, complicates the problem of maintaining stable populations of food chain organisms in habitats subject to heavy metal contamination.

HUBSCHMAN concludes by stressing that to evaluate properly the stresses produced by environmental change, we must consider the long term effects. Also, he says, the need for specific physiological parameters in this type of research is evident.

The ideas and whole approach to the problem in this study are very encouraging since in the past it does seem that the points brought out here have been somewhat neglected, and it would seem that for certain animals, where the basic work of calculating Tlms etc. has been done, this further stage of research could well be undertaken. This study of HUBSCHMAN's is significant in that it opens up new and far-reaching pathways in a field that at times gives the impression of becoming static in its progress and rather cramped and limited in its horizons.

Another very interesting study which appeared in 1967 is that by CHIARANDINI, STEFANI and GERSCHENFIELD on the inhibition of membrane permeability to chloride by copper in Molluscan neurones. This work starts from the observation that when 10^{-5} molar copper sulphate is added to the Ringer solution bathing the outer surface of the abdominal skin of a frog it produces a dramatic reduction in the permeability to chloride. Frog skin has a very complex structure and the copper sulphate may have acted on many possible sites, so these workers decided to see whether copper sulphate had the same effect in a simpler system such as a neuronal membrane. It was found that copper sulphate practically abolishes the Chlorinergic IPSP and the ACh potential in snail H-neurones, and that this effect was due to a reduction of the membrane permeability to chloride ions. Work is also described in this paper on neurones from the isolated perioesphagic ganglionic ring of Cryptomphallus aspera. These were affected in a way similar to that described above and it was further found that this effect was reversible, and that the amplitude of the IPSP, the ACh potential and the resting potential recovered after prolonged washing in snail Ringer solution.

This study opens up an entirely new aspect of heavy metal toxicity, i.e. the study of the effects of heavy metals ions to the basic membrane physiolcgy of cells. This could be a highly revealing and exciting line of attack and work on the effects of heavy metals on active transport systems such as the sodium pump, which are basic and vital mechanism in cell physiology, could yield some very meaningful results. At present the techniques used in

this type of neurophysiology are probably good enough to make this angle possible in practical terms and it seems lamentable that more workers on this field have not taken this up to any great extent.

This review of the literature will conclude with the three review papers of SPRAGUE entitled 'The measurement of pollutant toxicity to Fish'. These papers appeared in 1969, 1970 and 1971 and are an attempt at a summing-up of the present situation as regards methods of bioassay and the interpretation of results from these bioassays. These review papers are therefore essentially concerned with the practical side of things as they have manifested themselves in recent years.

Paper 1 in this series is entitled 'Bioassay methods for acute toxicity' and here SPRAGUE describes various methods for measuring lethal levels of pollutants for aquatic organisms, and is at his most practical in giving a graph for estimating partial replacement times for water in tanks of continuous flow tests. He is at his most useful in giving a section on the terminology of this work which at times becomes nightmarish, and in urging the use of a standard terminology. The merit of this to both research workers and to prospective reviewers is self-evident. SPRAGUE also proposes a 'standard method' for toxicity testing starting with an estimation of the median lethal time,and describes various alternative handlings of this data.

As SPRAGUE has intended, this review will be of value for some time yet to 'students or scientists entering the field of toxicity tests with aquatic organisms'. It will go a long way towards

clearing up initial misunderstandings and possible bewilderment of the worker just entering this field and the extensive bibliography will be a great aid to getting into the literature.

The second paper (1970) is concerned with 'Utilizing and applying bioassay results'. This carries on logically enough from where Paper 1 left off and is a review of methods of handling data obtained from the toxicity test. Also SPRAGUE discusses findings concerning the effect of complicating factors such as temperatures and water hardness on the toxicity of a substance as well as the toxicity of mixtures of toxicants. As in part 1, SPRAGUE does not miss the opportunity of stressing the importance of establishing a standard terminology for the effects of two or more toxicants acting simultaneously. An interesting theoretical aspect is also described whereby it is possible to predict the toxicity of mixtures of two or more pollutants on the basis of chemical measurements. This is obviously of great practical importance.

Returning to the subject of complicating, or, as SPRAGUE calls them, modifying factors, SPRAGUE describes more advanced techniques which are being used in the processing of data obtained from tests incorporating variables other than concentration. Among the examples which SPRAGUE cites is McLEESE's by now classic three-dimensional graph which appeared as far back as 1956. Here a 'response surface' is produced which defines 48-hr LC50s for combinations of three lethal components. This technique was employed for work on the Lobster but it does not seem to have been widely used. Further techniques which seem to have in common a

desire to extract the maximum possible information from the data are described and these form another very valuable section for reference for anyone interested in more modern techniques for dealing with toxicity test data. Also, autopsy methods, chemical and histopathological, are mentioned and briefly discussed.

Paper 3 is entitled 'Sublethal effects and "safe" concentrations'. Here SPRAGUE's theme is that if the actual modes of action, whether truly physiological or not, of toxicants were better understood it would be possible to predict with more accuracy their effects as pollutants. He points out that there is much knowledge already in existence in the medical sciences which could be transferred with great advantage to this field.

Also, how to assess and measure sublethal changes is discussed in this paper. Within fish these changes have been demonstrated by sensitive histopathological and biochemical techniques, but even these leave us in ignorance of the ecological implications. The effect on growth rate is easy to measure (in fish) but, as SPRAGUE points out, it is not always a sensitive indicator of incipient toxicity. Swimming speed is also insensitive since it is often less affected by toxicants than might be expected. Respiration rate, behaviour and feeding are also discussed but SPRAGUE suggests that reproduction seems to be one of the most sensitive of chronic or sublethal responses which is meaningful in ecological terms. SPRAGUE advises that the reproductive response to toxicants should be tested wherever practical to do so.

In this paper SPRAGUE gives us his concept of the overall scope

and aim of bioassay work by saying that the ultimate method of bioassay work is an ecological survey of a polluted habitat and that laboratory bioassays are inadequate if they do not explain the results of field bioassays in polluted waters.

SPRAGUE discusses the term "safe" level, which in his review he uses in a loose way to mean 'the concentration of pollutant which does not have an adverse sublethal or chronic effect on fish'. As SPRAGUE points out, here the term "safe" has no meaning in absolute terms but as a 'statistic' whose value is empirically determined as the result of an experiment'.

SPRAGUE concludes his review by pointing out that there is a continuing need for critical reviews on individual pollutants.

In these three reviews we are given a panoramic view of the work which has been done in this field. They deal almost exclusively with fish but they have been included here because they contain all the basic thinking, theory and methodology which has grown up in this field since the days of RINGER. Also they underline and bring out clearly themes which have become important aspects of this type of research such as the concepts of sublethal effects, safe concentration and a need to correlate more closely laboratory and field work. All these papers contain a comprehensive list of references which should, in themselves, be of value to anyone interested in this field since they bring together the work from several disciplines in one convenient work of reference.

Chapter 2

GENERAL BIOCHEMISTRY AND TOXICITY OF COPPER

DISTRIBUTION

Research into copper and any relationship it may have had with living organisms seems to have its beginning in the work of MIESSNER who, in 1817, showed that copper was actually a constituent of plants. In a later study carried out in 1830 SARZEAU detected copper in numerous plants and determined the amounts quantitively. DESCHAMPS, in 1848, was able to demonstrate that a relationship existed between the copper in plants and that in the soil on which they were grown, and in 1868 CHEVREUL suggested that copper was quite widely distributed in organic matter. Lastly, in this group of early workers, LEHMANN deserves a mention for his 1895 review of all the then known facts regarding the distribution of copper, and for his own analyses which added more information and data to contemporary discoveries.

It will be noted that the dominant feature of this early work, apart from the interesting predominance of workers of French nationality, is that all these investigations attribute no definite function to the presence of copper inside living things. Generally, the assumption was made that the occurrence of this metal was accidental and of no real biological significance. This approach, with its preoccupation with, first, the detection of copper in a wide range of organisms, and then the determination of the amounts present, will be seen to persist in the work carried out in this field for some time after these originating studies.

During the first two decades of the twentieth century other workers became involved in the determinations of copper in the

tissues of plants and animals. Most notable of these are MAQUENNE and DEMOUSSY (1920), GUERITHAULT (1920) and FLEURENT and LEVI (1920). All these workers established without question the universal distribution of copper in plant life. MAQUENNE and DEMOUSSY found 30-40ppm copper in all the plant material they analysed and even went as far as to suggest that copper must be an essential element in plant metabolism. The fact that it was found mainly in the more active organs of plants, e.g. young shoots and leaves, led these workers to conclude that copper aided in some way the vital functions of the plant. It is interesting to note that this suggestion of a metabolically active role for copper in plants was made after the universal distribution of copper in plants was well established, and early workers were forced to abandon the not very satisfying idea of a purely accidental presence of copper. This would seem to be the broad pattern of events, since it is difficult to see why this suggestion of a metabolic role should have come with such, at least apparent, suddenness. The fact that copper was being detected in areas of greatest activity was no doubt a clue, but this, too, could have been explained as an 'accident' arising from a greater general metabolic turnover in these areas.

Thus far there does not seem to have been much of an attempt at a suggestion concerning a <u>particular</u> role which copper could play in living organisms, although there is a speculative suggestion put forward by GUERITHAULT and BERTRAND in 1920 that copper should be placed in the group of catalytic elements found in plants.

Although much of this early work was directed towards studies of

copper in plants, other workers soon became involved with parallel studies with animals. The fact that marine animals contain copper in comparatively large amounts was established early. In 1847 HARLESS detected copper in Eledone and Helix Pomatia, and showed that it did not exist as a free salt but in combination with blood proteins (Haemocyanins). In retrospect we can now look back at this discovery as quite an important breakthrough and it is somewhat surprising that it was not made much of at the time. What did appear to happen was that workers energetically turned their attentions to more determinations of copper content in the bloods of marine animals. The following table shows the relative distribution of copper in the bloods of various animals (not all marine) and is taken from the review which DHERE wrote in 1915 in which he summed up these early investigations.

TABLE 3 Copper contents of bloods containing haemocyanins

Animal	Copper mg per 100cc
Octopus vulgaris	23.5
Sepia officianilis	23.7
Helix pomatia	6.5 - 7.5
Astacus fluviatilis	7.0
Palinurus vulgaris	9.7
Homarus vulgaris	10.0
Cancer pagurus	6.0
Carcinus maenas	9.0
Maia squinado	3.5
Squilla mantis	6.1

It did not escape the notice of these early workers that if these animals are obtaining this copper from sea water then they must be concentrating it to a very great extent, since sea water contains only 10mg of copper per cubic metre (ATKINS 1932). There must, therefore, be active uptake of copper against a considerable concentration gradient by these animals. Lists of copper concentrations like that shown above must have provided strong clues pointing towards an important physiological role for copper. Animals usually show great physiological economy and very rarely use up energy to take up ions from their environment if they are not essential to their metabolism.

Other workers were carrying out studies on a fairly wide range of invertebrates (mainly marine) and producing tables of copper concentrations of the type shown above. ROSE and BODANSKY in 1920 produced one showing all the animals in which copper was known to occur and pointed out that the data that far accumulated were chiefly from arthropods and molluscs, two phyla in which haemocyanin is widespread. The significance of this was perhaps not not fully grasped at the time since the structure of the haemocyanin molecule was not then known in great detail. However, ROSE and BODANSKY went on to make more quantitative studies on copper in no less than 35 marine animals, most of which, significantly, were fish. This seems to be the first study made of copper in relation to vertebrates. The results showed that copper was present in all of the fish they studied with the exception of the Pigfish. The amount of copper in each of the individual samples averaged about 2.5mg per Kilo, i.e.

very much less than the amounts found in marine invertebrates.

With this extension of the study of copper distribution to vertebrates a general pattern of overall copper distribution in the animal kingdom was beginning to emerge. This pattern seemed to show that the amounts of copper in the animals examined was often found to diminish as the animals ranged from the comparatively simple to the more complex levels of organization. ELVEHJEM, writing in 1935, pointed out that when animals having haemoglobin and not haemocyanin are reached copper was found at much reduced levels. The two exceptions to this emerging 'rule' were Aurelia and Physalia, which were found to contain the same amount of copper as fish. These animals, however, have a much higher moisture content than do vertebrates and most other invertebrates. When two mammals, the Whale and Sea lion, were examined no copper at all could be detected in their tissues (SEVERY, 1923). This last study seemed to support the then widely held view that copper was not a definite constituent of higher animals, even though as early as 1901 THUDICUM had been able to demonstrate that copper was present in the human brain. This work was not held to show unequivocally the presence of copper, however, since it was argued that the tissue which THUDICUM examined may have come from subjects exposed to copper poisoning. However, in 1921 BODANSKY verified that copper was indeed present in the human brain. The quantities which he was able to detect were in the order of 3.6 to 6.0mg per Kilo. In one foetal brain he found 6.8mg per Kilo. Another study made by McHARGUE in 1925 revealed that the liver contains a high concentration of copper, especially when one

from a young animal is analysed.

Since the time of these studies copper has been accepted as being present in tissues from all animals as well as in plant tissues.

Physiological role

It has already been pointed out that as early as 1847 it had been shown that copper in the bloods of Elodone and Helix existed in combination with the blood proteins. The first suggestion of an actual physiological role for copper comes from BLASIUS who, in 1866, speculated that copper played some role in the respiration of lower animals. He based this idea on the consideration of the constancy with which copper was found in the bloods of lower animals. If this was an inspired guess it was not a bad one. In 1867 the compound present in the blood of molluscs and certain arthropods was considered by BERT to be a definite respiratory pigment. Evidence for this came partly in 1878 when FREDERICQ isolated the copper containing protein of the octopus (O.vulgaris) and gave it the name Haemocyanin. Based on this work he went on to state that a copper compound analogous to haemin could be separated from this protein by acid and even went further to predict that the equilibrium between oxygen and this protein was governed by factors similar to those affecting haemoglobin.

Before haemocyanin was isolated a number of workers were interested in the copper protein in various invertebrate bloods from the point of view of a protein which would be analogous in function

if not in structure to the haemoglobin of higher animals. This interest prompted several workers to determine the oxygen carrying capacities of the bloods of different species. QUINQUAND (1873) and JOLYET and REGNARD (1877) were the first to make measurements of this nature. It is interesting to note that HALLIBURTON, in his paper of 1885, states that JOLYET and REGNARD were the first to suggest that copper was united to a protein. Measurements of the oxygen affinities of various bloods containing copper followed by other workers and a review of these has been written by DHERE (1919). Practically all the figures range from 1 to 5cc of O_2 per 100cc of blood, and one point that emerges from these studies is that the bloods from different species have unlike oxygen affinities. For Cephalopods in general it is relatively high, in Crustacea it is not as high, and some decapods such as Cancer it is low.

HENZE in 1901 prepared haemocyanin in crystalline form for the first time and found that, unlike haemoglobin, it could not be broken down into a protein and a protein-free component. According to HENZE the haemocyanin of Octopus contains 0.38% copper and 1g of the protein combines with about 0.4% of oxygen. This works out at about $\frac{1}{3} - \frac{1}{2}$ of that for haemoglobin. Further work by ALSBURG and CLARK in 1910 used the blood from the horseshoe crab, Limulus polyphenus, and their work led them to suggest that the protein of this blood differed from that of Octopus. Limulus was found to contain only 0.28% copper, but in 1928 REDFIELD, COOLIDGE and SHOTTS gave a figure of 0.173% for Limulus.

This relation of the oxygen combining power to copper content of

different bloods containing haemocyanin has attracted much attention since FREDERICQ first suggested that this compound acts as an oxygen carrier. Many workers, including DHERE, felt that there must be an at least general correspondence between the oxygen capacity and the copper content of the various invertebrate haemocyanins. In 1907 MENDEL and BRADLEY showed that the blood of Octopus contains 18-23mg Cu per 100cc, whereas Helix pomatia contains only 6.5-12.5mg per 100cc. From these figures they drew notice to the obvious relation between amount of copper and the differing levels of activity of these two animals, i.e. Octopus is a highly active Cephalopod and therefore requires more oxygen; on the other hand, Helix is rather inactive and consequently has a lower oxygen demand. It therefore has less copper in its blood, giving it, presumably, a lower oxygen capacity. This simple relation between the copper in the blood and oxygen affinity came under question when, in 1914, ALSBERG and CLARK obtained only 0.5 volume % of oxygen from Limulus blood. This extremely small amount, they argued, could be explained by the solubility of the oxygen in the blood itself. They suggested that the oxygen of oxyhaemoglobin was made available by diminished pressure alone, but that copper acted in some hitherto unknown catalytic manner in the transfer of oxygen from the blood pigment to the tissues.

BERGEMANN in 1924 was the first to study the actual amount of oxygen held in physical solution by the blood of invertebrates. He precipitated the proteins of the blood in his determinations, but later work by STEDMAN and STEDMAN (1925) and REDFIELD (1925) provides

figures for the solubility coefficient of oxygen in the unchanged blood. The method used by these workers was to determine the oxygen content at two oxygen pressures both of which were sufficiently great to saturate the haemocyanin completely. The latter workers found the solubility coefficient to be about 0.022% for the bloods analysed and that in nearly every case the solubility of the oxygen was lower in the blood than in sea water. It was then demonstrated that Limulus blood has a definite oxygen capacity which is much greater than can be accounted for by solubility alone and varies from 1.66 to 0.14 volume %. Further figures for the bloods of Loglio peali, Busycon canaliculatum, Cancer irroctus and Calinctus sapidus establish a definite oxygen capacity above that held in physical solution for the haemocyanins of these animals.

The most important question which emerged at this time concerning the actual function of haemocyanin in respiration centred around the study of the equilibrium between oxygen, haemocyanin and oxyhaemocyanin and the effect of carbon dioxide on this equilibrium. BERGEMANN (1924) and REDFIELD et al (1926) had shown that the same phenomena affect the gaseous exchange in bloods containing haemocyanin as in those containing haemoglobin. REDFIELD and GOODKIND in 1929 pointed out that the reciprocal effects of carbon dioxide and oxygen on the respiratory properties of squid haemocyanin accounted for $\frac{1}{3}$ of the respiratory change. PARSONS and PARSONS (1923) had already studied the transport of carbon dioxide by the bloods of invertebrates and concluded that haemocyanin functions in the transport of carbon dioxide in the same way as haemoglobin. These results were confirmed in 1925 by REDFIELD et al, who summarized the work as follows:

'Studies of the conditions of equilibrium between oxygen, carbon dioxide, haemocyanin and oxyhaemocyanin show that the properties of haemocyanin of each of these species are distinctive, but that all these proteins function in transport of oxygen and carbon dioxide according to the same physico chemical principles as obtained in the case of haemoglobin, although the reciprocal relation between oxygen and carbon dioxide (the Bohr effect) is reversed in the case of certain bloods containing haemocyanin'.

In BERGEMANN's 1924 work he found that the bloods of both snails and crabs combine with oxygen in a definite ratio to the copper present. This amount in combination suggested to BERGEMANN that one atom of oxygen unites with each atom of copper present in the haemocyanin molecule. REDFIELD et al.(1928) give the combining ratios for the bloods of nine species of animals which demonstrated that oxygen combines with haemocyanin in a simple stoichiometrical proportion, one atom of oxygen to one atom of copper. These studies, therefore, showed that all haemocyanin (i.e. all those studied) containing copper seemed to unite with oxygen in a definite, fixed ratio. It was concluded from this that there is a similar copper group common to haemocyanins from all the different species studied.

The first suggestion as to what this prosthetic group might be seems to have come from CONANT et al. in 1934. These workers put forward the hypothesis that the prosthetic group of Limulus haemocyanin is a complex copper salt of an unknown sulphur compound and a polypeptide, consisting of one molecule each of leucine and

tryosine, and three of serine.

These and other early studies have served to establish haemocyanin as the characteristic blood pigment of gastropods, cephalopods, molluscs, crustaceans, arachnids and the Xiphosura. Further work has established other properties of haemocyanin such as its colloidal nature and its molecular weight of 1,000,000. Haemocyanin is not unique as a copper protein although, so far, it is the only one to which an oxygen-carrying capacity has been ascribed (FLOREY, 1968). Copper is also found in a variety of enzymes which will be discussed briefly later.

Copper in higher animals

As already mentioned, workers such as ROSE and BODANSKY had detected copper in fish and the even earlier study of THUDICUM had shown that copper is present in other vertebrates in small concentrations. The highest concentrations of copper are generally found in liver tissue (6.6μg/g) and brain (5.4μg/g). In rat liver copper has been found to be distributed among the subcellular factions as follows:

soluble faction	64%
mitochondria	8%
microsomes	5%
nuclei & debris	20%

Copper had been discovered in human blood serum by WARBURG and KREB in 1927 in an amount approximating to 0.0017mg copper per cc. Also, three workers report copper as occurring in the blood sera of

dog, cat, rat, guinea pig, frog, chicken and goose. In all these cases copper was also found in association with the serum proteins of these animals.

As with the case of copper in lower animals, the work which resulted in an accumulation of quantitative data for a series of animals gave way to speculation concerning the nature of this copper and its function. Early work in this field seems to have started with dietary experiments in which copper was excluded from the diet. The result of copper deficiency as manifested by the health of the test animals was used as a clue to the possible deeper, physiological role of copper. The pathology associated with copper deficiency was found to include anaemia in all the species studied (HART, STEENBACK, WADDELL and ELVEHJEM, 1928), neonatal ataxia or sway back in lambs, achromotricha or lack of pigmentation, and connective tissue defects including bone disorders and cardiovascular failure. In man copper may be the cause of Wilson's disease. Here, a mechanism which regulates copper retention fails to function and copper accumulates in certain tissues, particularly brain, liver and kidney, while plasma levels are lower than normal. Yet another effect of copper deficiency is that the absorption of iron from the gastrointestinal tract of swine is impaired. It may be that copper is needed for the release of iron from tissues, including the intestinal mucosa which accumulates iron in copper-deficient animals.

Copper occurs in animal tissues in the cupric form and it may be that the cupric ion serves as an oxidative catalyst, e.g. in the oxidation of ascorbic acid by molecular oxygen to form dehydrascorbic

acid. The catalytic activity of the cupro-protein ascorbate oxidase is 1000 times as great as that of an equivalent amount of cupric ion.

Essentially, it seems, all copper containing metalloenzymes are concerned with catalysis of oxidation-reduction type reactions in which oxygen is the electron acceptor. Table 4 is a list of the better known copper metalloenzymes (taken from ODELL and CAMPBELL, 1971).

TABLE 4 Copper metalloenzymes

Enzyme	Cu. content %	Cu. content g.atoms/mole.
Cytochrome oxidase	Cu:HAGME = 1	3
Ceruloplasmin	0.32	8
Ascorbate oxidase	0.34	8
Tyrosinase	0.20	1
Laccase	0.25	4
Galactose oxidase	0.85	1
Uricase	0.05	1
Dopamine β -hydroxylase	0.05	2
Diamine oxidase (pea seedling)	0.09	1
Diamine oxidase (kidney)	0.07	2
Plasma amine oxidase	0.09-0.1	4

Of these enzymes cytochrome oxidase seems to have been given the most attention. This is a lipoprotein in nature and is bound to mitchondrial membranes. It was first recognized as a haemoprotein (i.e. containing iron only) and the earliest reference to cytochrome, or, rather, to a compound which has only subsequently been known by that name, is by MACMUNN. In his work of 1884-1886 MACMUNN described

a respiratory pigment which he found in muscles and other tissues of a very wide range of animals. To this pigment he gave the names myohaematin and histohaematin, and after carrying out further studies on this pigment he was able to conclude that myo-and histohaematin were respiratory pigments different and independent from haemoglobin or any of its derivatives. This was more or less immediately refuted by other workers, principally LEVY (1889) and HOPPE-SEYLER (1890), and for a time MACMUNN's work appears to have been forgotten or ignored by other workers. It was not until KEILIN's work in 1925 that MACMUNN's ideas were in fact vindicated. KEILIN found that myo-or histohaematin not only existed but had a much wider distribution and importance than were anticipated even by MACMUNN. KEILIN went on to argue that since this pigment is not confined to muscles and tissues but exists also in unicellular organisms, and, further, that there was no evidence that it was a simple haematin, the names myo- and histohaematin were inappropriate and misleading. KEILIN believed that the pigment was not simple but was a complex formed of three distinct haemochromagen compounds. He therefore proposed the name cytochrome, meaning 'cellular pigment', a name which at the time was intended to stress not only its wide distribution but also the fact that its chemical make-up was still unknown. It is by this name, however, that it is still known today.

KEILIN found that the highest concentrations of cytochrome were to be found in the thoracic wing muscles of flying insects, striated muscles of mammals and birds, and baker's yeast. Also, he produced evidence that cytochrome actually consists of three haemochromagen

compounds (a', b' and c'), two of which (b' and c') he claimed had a haem- nucleus (iron-pyrrol compound) similar to that of haemoglobin. It was not until 1938 that KEILIN detected copper in cytochrome c oxidase preparations, this time in collaboration with HARTREE. The early evidence suggesting that copper may be a constituent of cytochrome oxidase was not provided only by the analytical results of KEILIN and HARTREE but also by independent nutritional studies. In such studies of their own KEILIN and HARTREE observed that the cytochrome oxidase activity in the heart muscle of copper-deficient rats, pigs, cattle and sheep is greatly reduced. Also, they observed that heart preparations contained copper and postulated that cytochrome oxidase is, in fact, composed of cytochrome a and a3. There is now no reasonable doubt that cytochrome oxidase contains copper which exists in a ratio of 1:1 with haeme a. The concept of two compounds, a and a3, has been challenged, however.

The nature and function of copper in cytochrome oxidase is not entirely clear. Only 30-40% of the copper is accounted for, as cupric copper and copper can be removed from cytochrome oxidase with loss of enzymatic activity. The apoenzyme contains about 45% of the original copper and still retains about 15% of its activity. When copper is restored the activity is regained, thus part of the copper in the enzyme undergoes a valency change during catalytic activity and part is essential for enzyme activity.

In 1950 WAINIO et al. presented analyses of their deoxycholate-solubilized purified cytochrome oxidase preparation. This data indicates a relationship between the absorption band at 601 mμ and

the copper content of the preparation, whereas no such relationship was apparent for the iron content. These findings imply that cytochrome oxidase may be a copper porphyrin compound. Subsequent work by PERSON and WAINIO and EICHEL in 1953, and DANNENBERG and KIESE in 1952, left no doubt that the porphyrin moiety of the enzyme, considered as a prosthetic group, did not contain a copper but an iron nucleus. It was, however, left open as to whether the copper present was actually a constituent of the enzyme or merely a contaminant. Thus it is seen that a hitherto fairly simple picture of copper in cytochrome was somewhat complicated by findings using more sophisticated techniques than had been available to earlier workers.

Repeated efforts failed to establish copper as a meaningful constituent of cytochrome c oxidase. GREEN et al (1956) found that copper was concentrated in preparations of NADH oxidase, a submitochondrial faction containing the cytochrome oxidase component, and this was confirmed by WAINIO et al as well as other workers. In 1965 BEINERT reviewed the weight of evidence then available and according to this he stated that the 'unit' of cytochrome oxidase consists of one cytochrome a and one cytochrome a_3 and each cytochrome is associated with one copper molecule. The copper atom associated with cytochrome a is cupric and the state of that associated with cytochrome a_3 is unknown. According to BEINERT the unit as a whole is able to take four electrons on reductive titration and kinetic studies indicated that the cytochromes as well as one of the copper species - presumably that associated with cytochrome a - participate in the enzymatic reaction as electron carriers. The evidence that

copper is indeed an integral constituent of cytochrome oxidase with a role in the transference of electrons comes mainly from the following sources:

1. That animals fed on diets deficient in copper exhibit a subnormal level of cytochrome oxidase (COHEN and ELVEJEM,1934; SCHULTZ, 1939 and 1941; GALLAGHER et al,1956; GUBLER et al,1957; MILLS and WILLIAMS,1962).
2. Purified preparations of cytochrome oxidase from mammalian sources contain copper. When properly characterized these preparations possess an equimolar ratio of copper and haeme compounds.
3. During purification there is a parallel increase of copper and enzymatic activity.
4. Copper undergoes oxidation and reduction and inhibitors which block the reoxidation of the haeme components also prevent reoxidation of the copper.

Although such evidence would seem to point strongly to a real role for copper in this enzyme, the situation is still far from unequivocal and the hypothesis is still, to a certain extent, a controversial one.

This brief survey of the biochemistry of copper will be concluded with some very brief notes on other copper metalloenzymes.

Ceruloplasmin (found in blood plasma)

The role of this is not yet known with certainty but it does possess oxidase activity and is classed as a metalloenzyme (O'DELL and CAMPBELL,1971). Its more critical function may relate to copper

balance and transport. A postulated role for it is promotion of iron utilization by stimulation of iron oxidation and transfer in saturation.

Tyrosinase

This enzyme occurs in both plant and animal tissues. It appears to catalyse two types of reaction, hydroxilation of mono-phenols and dehydrogenation of catechols. Copper is very easily dissociated from this enzyme.

There are, in addition to the copper metalloenzymes listed in Table 4, several less well defined copper metalloproteins. These are shown below in Table 5 :

TABLE 5

Protein	Source	Cu. content %	Cu. content g.atoms/mole.
Haemocuprein	bovine erythrocytes	0.34	2
Erythrocuprein	human erythrocytes	0.32-0.36	2
Hepatocuprein	mammalian liver	0.34	-
Cerebrocuprein	human & bovine brain	0.29	2
Mitochondrcuprein	human & bovine neo-natal liver	3.0-5.0	-
Haemocyanin	snail	0.23	32

These proteins constitute well over half the copper found in the respective tissues from which they are isolated. O'DELL and CAMPBELL (1971) suggest that this fact points to their chief function being one of copper storage.

The physiological role of copper may be summarized in the following way:

1. In lower animals it has a respiratory function through its presence in haemocyanin. In this respect haemocyanin is a unique cuproprotein in that it performs the specific physiological function of oxygen transport.
2. In mammals copper is a necessary trace element in the building up of haemoglobin. Most of the evidence for this was derived from dietary studies.
3. Copper in association with cytochrome is a necessary component and has a definite role in oxygen transport. This, however, is still open to some question, and elucidating of details. It is certain, however, that copper is a genuine constituent of the molecule, held by a specific linkage to the protein (LEMBERG, 1969).

A recent study of the haemocyanin from Octopus vulgaris Lam. (SALVATO, GHIRETTI-MAGALDI and GHIRETTI, 1974) has thrown some light on the structure of this molecule. By using potentiometric and spectrophometric titration techniques these workers showed that each of the 2 copper atoms in the functional sub-units is bound to 4 non-carboxyl groups. FELDMAN and WEE (1974) were able to show that the cupric ion has a very great catalytic effect on the nonenzymatic dephosphorylation of ATP. Copper has also been shown to catalyse the oxidation of oxyhaemoglobin even at very low concentrations (RIFKIND,1974). This is interesting, especially since it appears that other metal ions have no significant effect even at much higher concentrations. RIFKIND was also able to demonstrate that haemoglobin has a high copper (11) affinity which suggests that the oxidation involves copper bound to haemoglobin. In the light of

these findings it is suggested that copper is actually responsible for a large part of what has been hitherto thought of as autoxidation.

Toxic action of copper

PETERS, SHORTHOUSE and WALSHE (1965) have pointed out that the toxic action of copper is of theoretical interest as it is a normal component of cytochrome oxidase and other proteins. This, however, also has a practical bearing on the origins of disturbance in the central nervous system (this is well known in cases of Wilson's disease where patients accumulate copper). Also, in an earlier study PETERS and WALSHE (1962) had found that copper in trace amounts inhibited the respiration of mitochondria by blocking pyruvate oxidation. In the case of central nervous system disturbance extremely small amounts of copper are involved, since most of the copper is bound to the insoluble neurokeratin and less than 5μg takes part in the toxicity. Further, cupric ions of concentrations as low as 10.6μ inhibit the membrane ATP ase of pigeon brain. In this connection it has also been discovered that heavy metals such as copper have the power to inhibit facilitated transfer and to increase permeability to K^+ions (DAVSON,1970).

Most of the detailed work on the toxic effects of copper has been carried out with vertebrate animals but there is some evidence that copper can inhibit the formation of urine in the malpighian tubules of Calliphora (BERRIDGE,1969). Here the tubules utilizing anions such as chlorides or nitrates are inhibited by copper and the rate of urine formation declines. Complete inhibition of urine formation occurs in two hours.

*

As SPRAGUE (1971) has pointed out, 'understanding the physiological action of a toxicant is the key to predicting important sublethal effects'. If such an understanding is to come about there must be a broader attack made on the subject of copper toxicity in relation to invertebrates and toxicity. SPRAGUE suggests approaches such as histopathology, histochemistry, haematology, biochemistry and physiology, may be used to determine the mode and severity of the action of a toxicant, and although he was referring to studies on fish toxicity these approaches are also applicable to studies with invertebrates.

As a conclusion to this chapter Table 6 has been compiled from the work of various workers to show the toxicity of copper in terms of threshold concentrations to a wide variety of animals. As can be seen, the toxic effect of this is variable and does seem to be fairly related to the size of the organism, although other factors such as the possession of an impermeable integument (as in the case of the Crayfish) must play a part. In the case of fish the method of poisoning (asphyxiation due to copper forming an impermeable barrier with mucus secreted on the surface of the gills) may account for low threshold concentrations, since fish must be vulnerable to even low concentrations of copper. It is interesting also to note that there are discrepancies between the threshold concentrations arrived at by different workers for the same organism. This is probably due to many factors including the use of different experimental set-ups, different states of the animals used and general lack of standardization in the use of the toxicity test.

TABLE 6 THRESHOLD OF TOXICITY OF COPPER TO VARIOUS ORGANISMS

Organism	Threshold concentration as mg Cu/L.	Reference
Orconectes rusticus (Crayfish)	3.0	Hubschman, 1967
Trout	0.09	Anon., 1950
Carp	0.22	
Sucker fish	0.22	
Catfish	0.26	
Pickerel	0.26	
Gold fish	0.33	
Perch	0.44	
Blackbass and bluegill	0.53	
Sunfish	0.89	
Scandesmus	0.15	Bringmann and Kühn, 1959
Daphnia	0.10	Bringmann and Kühn, 1959
Escherichia coli	0.08	Bringmann and Kühn, 1959
Microregma	0.05	Bringmann and Kühn, 1959
Neosphaeroma oregonensis	0.13	Klock and Pearson, 1959
Mytilus edulis	0.21	Klock and Pearson, 1959
Mercenaria enigmatica	0.21	Klock and Pearson, 1959
Polycelis nigra	0.31	Jones, 1940
Daphnia magra	0.06	Anon., 1950
Paramecium	0.00012	Ludwig, 1927
Frog tadpoles	0.0013	Dilling and Healey, 1926
Goldfish	0.12	Powers, 1917
Gammarus pulex	0.0013	Jones, 1937
Balanus balanoides	0.22	Clarke, 1947
Balanus eburneus	0.22	Clarke, 1947
Mytilus edulis	0.55	Clarke, 1947
Stone loach	0.20	Mackereth and Smyly, 1951
Taphius glabratus	0.05	Yager and Harry, 1963
Stickleback	0.03	Jones, 1939
Fathead minnow	0.05	Tarzwell and Henderson, 1960
Bluegill	0.20	Tarzwell and Henderson, 1960
Minnow	1.0	Liepolt and Weber, 1958
Brown trout	1.0	Liepolt and Weber, 1958

In general terms, what emerges from this table is that copper is toxic to a wide variety of organisms and in most cases the amounts which bring about these toxicities are small ones.

Chapter 3

THE ANIMALS

So that the reasons for certain usages in this investigation may be better understood it is necessary to give a brief description of the animals and their relationship to their environment at this point.

Ephemeroptera (ephemeros, living a day; pteron, a wing) are better known by their common name, Mayflies. As the name of the order to which they belong implies, many mayfly live only a few hours as imagines (sexually mature adults), but this peculiarity is compensated for by the lengthy nymphal life which may be as long as three years in some species of these insects. Eggs are laid in water and from these the nymphs emerge. These grow by shedding their skins, there being up to 40 such moults before full size is attained. Ultimately metamorphosis takes place and a winged form emerges from the final nymphal skin. This then undergoes a further moult (a feature which is unique to this group of insects) to produce the fully mature adult. This dies when it has mated and laid eggs. Thus the adult 'life' of the mayfly displays an extreme economy where the adult insect is virtually a reproducing 'machine' whose sole function is to produce, in the case of the female, fertile eggs. The gut is not functional and therefore the adults do not feed.

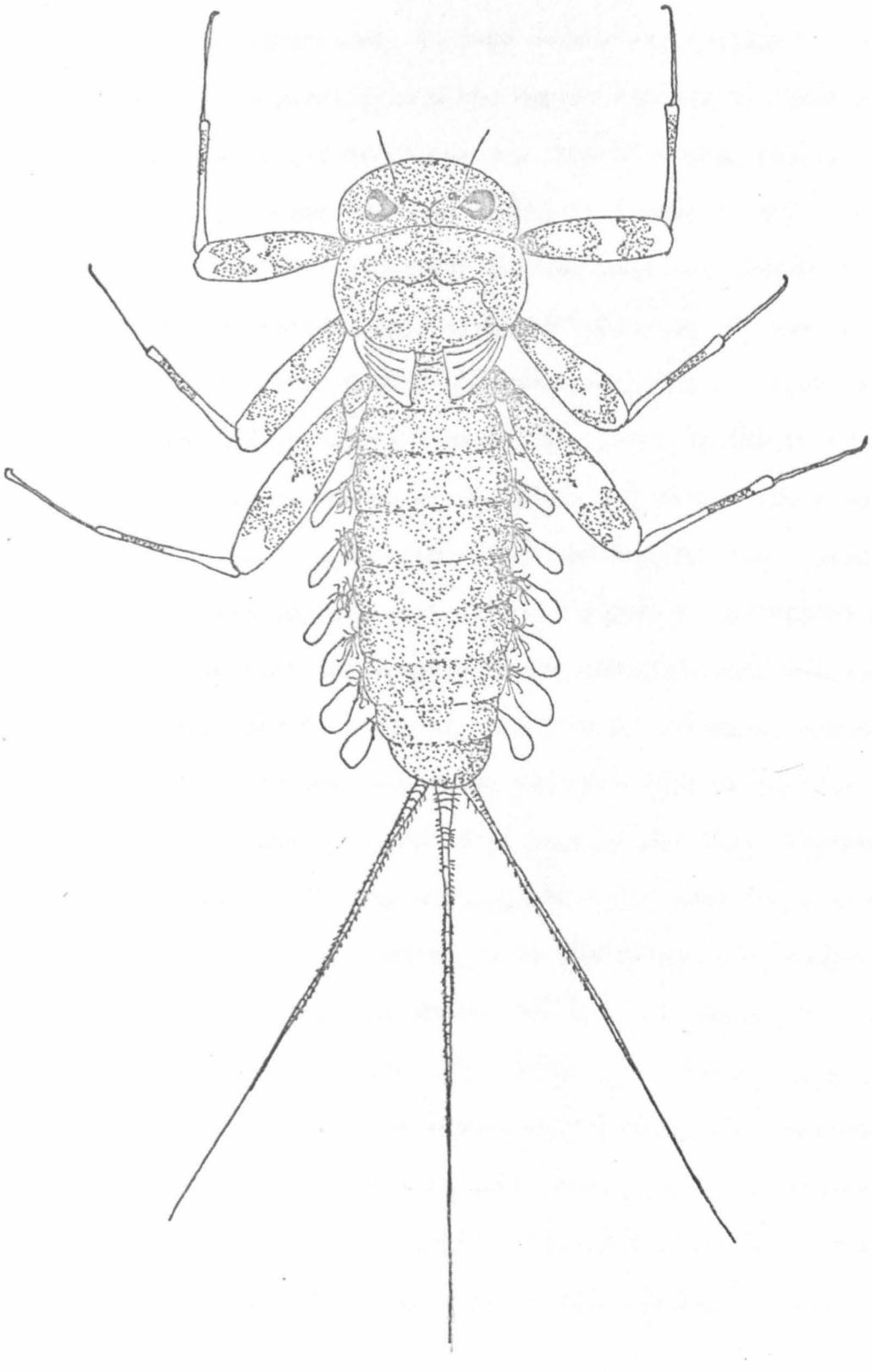
It is the nymphal stage of this life cycle that can be said to have a true ecology, i.e. a true relationship with their environment over an extended period of time. This stable relationship is a result of these insects having successfully come to terms with their environment in terms of having successfully adapted to their special ecosystem. One must, of course, bear this in mind when attempting to set up laboratory studies in which these insects are to be used as

test animals. More of this important aspect will be discussed after a brief description of the insects themselves.

Mayfly nymphs come in two main types of body shape. They are either broad with dorso-ventral flattening (i.e. an essentially flat body) or they are what can very loosely be described as the 'fish-like', i.e. their bodies are narrow and tapered towards the posterior, producing a rather streamlined form. The three species used in this study show both these two body shapes. As will be seen shortly, these shapes are intimately related to the physical environment. Fig. 6 is a drawing of the nymph of Ecdyonurus venosus which illustrates the flat type of body. Rithrogenia semicolorata, another species used in this study, is also of this type, although the flattening here is not so pronounced. The last species used is Baetis rhodani and this is typical of the 'fish' type of nymph.

These body shapes are directly related to the type of environment that these insects find themselves in in that they are designed to help the nymph keep its position in a sometimes fast current. All three species used here, and most other mayfly species, live in moving water and it is essential that they should be able to maintain their position in the current to avoid being swept away to a less favourable part of the river. The nymph with the flat type of body achieves this by pressing its body closely to stones so that its mass largely occupies the thin film of water just above the surface of the substrate in which the speed of the flow is at its minimum. In the case of B.rhodani and other nymphs of the 'fish' type, their body shape gives them swimming powers which, in

FIG 6 Ecdyonurus venosus x 18 (after Macan)



normal circumstances, enables them to swim or hold a position against a prevailing current.

This preoccupation with the maintenance of position in a stream is of importance, to some extent for feeding but more so in relation to respiration and the oxygen demands of these animals. A nymph living in a fast stream can live with less actual dissolved oxygen in the water than one living in a pool of still water. This is not because the fast water species need less oxygen but because in a fast-moving stream more oxygen is available to them as a result of the continual fresh supply of oxygenated water. Fast-water species often have higher oxygen demands than those living in still water and it is only by virtue of movement that water which may, in absolute terms, contain less dissolved oxygen than a stretch of still water, can satisfy the high oxygen demands of the nymphs living there. The fast water nymphs have therefore become adapted both structurally and in terms of behaviour to living in sometimes very fast-flowing streams from which they are able to utilise the necessary amounts of oxygen they require for their particular level of metabolism. If such species are swept away there is the possibility of being carried to and deposited into deeper and stiller reaches of a stream from which they would no longer be able to extract sufficient oxygen even though the level of actual dissolved oxygen may be the same or even higher than in the fast water reaches. This apparent paradox can be explained when one considers that an animal takes up oxygen from the water over the whole volume of water surrounding it. This, in a short time, causes an oxygen gradient to

be set up. If the rate of oxygen uptake is increased it will sooner or later reach a point at which the oxygen uptake becomes equal to the maximum diffusion rate possible in the water, i.e. the water immediately surrounding the animal now becomes depleted of oxygen to such an extent that it no longer can satisfy the animal's oxygen demand. This, and a remedy for this situation, can be shown graphically as follows:

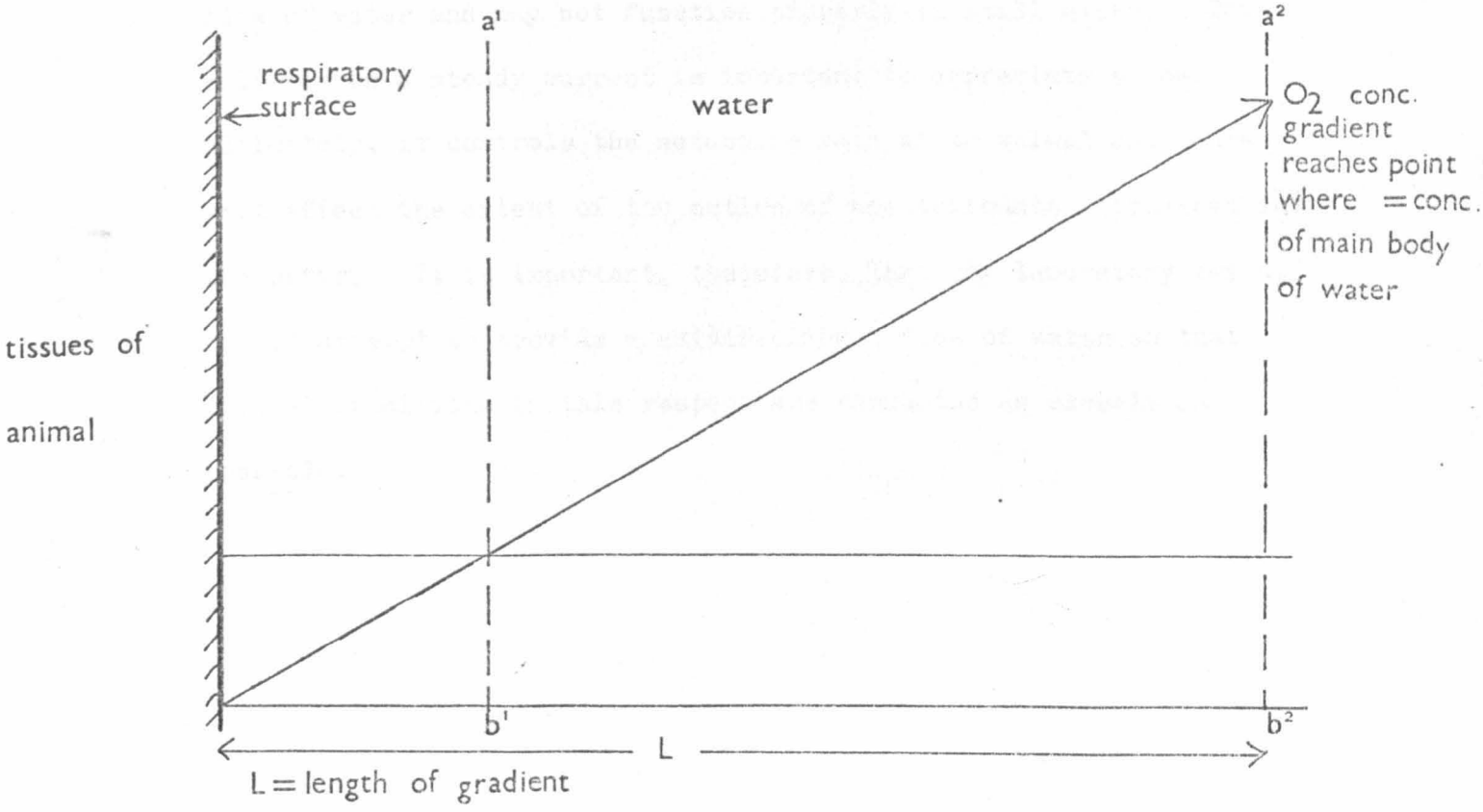


FIG 7

If the volume of water around the animal is moved so that the water of the outer concentration is brought in (so that L is shortened) the gradient is now shortened to a' b'. If this is done continually as it would be in a fast stream, the animal would then once more be able to satisfy its old consumption rate but in water which has a lower actual or absolute concentration of dissolved

oxygen. Alternatively, it can consume oxygen at a higher rate.

Thus, a fast-running stream is essential to animals with high metabolic rates since it increases the availability of oxygen if not the actual concentration. In the case of the mayfly, the movement of water around the immediate vicinity of the body is accentuated by the precisely coordinated movements of the abdominal gills. These movements are themselves, however, stimulated by a unidirectional flow of water and may not function properly in still water. This reliance on a steady current is important to appreciate since, ultimately, it controls the metabolic rate of an animal and hence will affect the extent of the action of any toxicants introduced into the water. It is important, therefore, that any laboratory set up should attempt to provide a unidirectional flow of water so that natural conditions in this respect are simulated as closely as possible.

ECOLOGY

No animal living in an ecosystem is entirely independent of the other organisms with which it shares that system. The fate of the population of one species may, and often does, have far-reaching effects on a population of, perhaps taxonomically, totally unrelated species. This phenomenon is due to a series of relationships which have a predator/prey organisation as their basis. These relationships range throughout the different types of animals inhabiting an ecosystem and are the basis of the complex interactions between animals and plants of different groups which are found in the so-called food chains or, more accurately, food webs. These webs are mostly of a very complex nature but very generally it is possible to represent them very simply by means of the food pyramid.

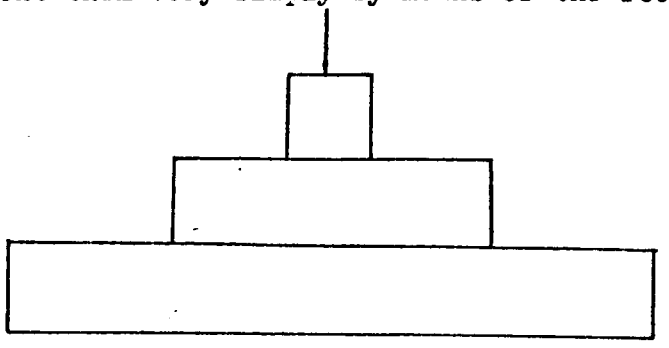


Fig.8 A Food Pyramid

These pyramids have their beginnings in autotrophic organisms which are able to utilize the energy in solar radiation to build up complex organic molecules. These are usually known, ecologically, as primary producers and form the lowest, and usually the largest, block of the pyramid. It is clear that a plant may be eaten by one

animal of a certain group which in turn becomes the prey of another group of animals and so on, thus forming a chain the links of which are made up of all the groups engaged in this predator/prey regime. At each of these steps or links in the chain energy is being transferred from one group to another. This is an inefficient process and some energy is lost at each step, hence the number of organisms is reduced with each energy transformation. This is an extremely simplified view of feeding relationships and is rarely found in this exact, simple form in nature. It can be seen, however, that if one group of organisms comprising one level of the pyramid were suddenly to disappear or have its numbers drastically reduced by some event outside the balanced ecosystem, this would affect not only the groups above this level but also those below.

Part of a food chain or web which include mayflies is shown in Fig.9 . From this, which is only a small part of the entire structure, one can begin to see how complex the organisation can be.

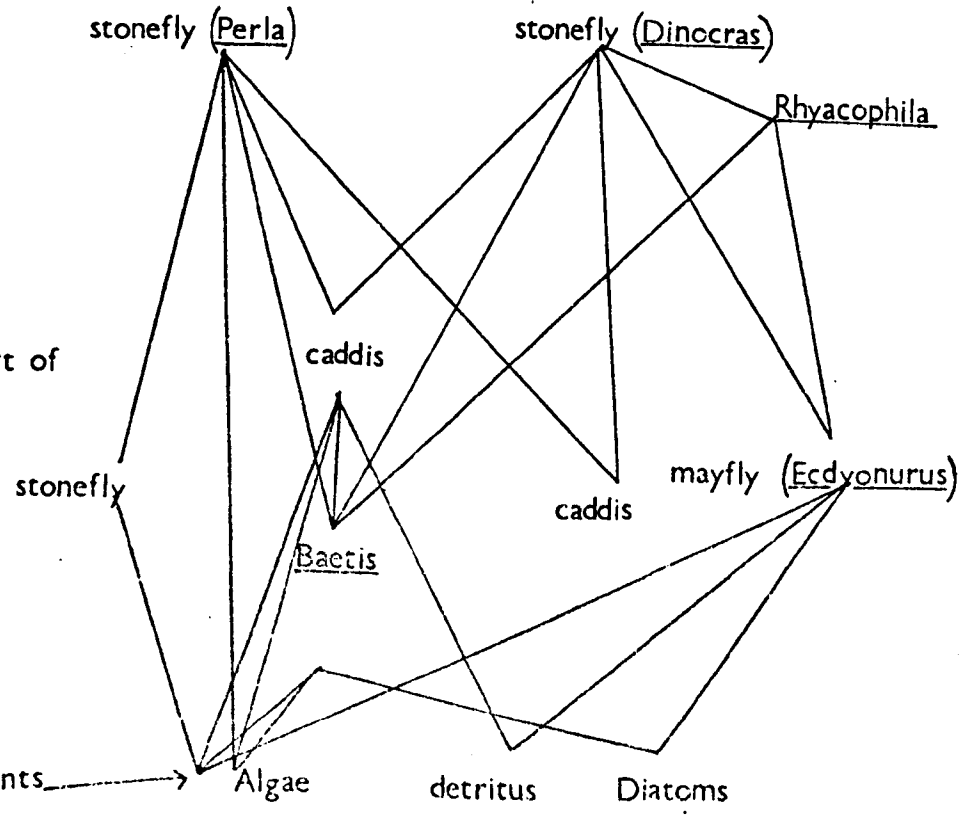


fig 9 part of a food web

To say that the animal groups which are in this system are not particularly dependent upon each other is at the very least a debatable point, and it is difficult to agree with Sprague when he suggests that there is no particular interaction (SPRAGUE, 1971).

As can be seen from the above figure, the mayfly occupies a position in the food web and as such will be preyed on by several carnivor types higher up in the web, these in turn will form the food of top carnivores which may have a certain economic importance (i.e. fish of various types). Mayflies, therefore, play an important part in such ecological webs, all the more so because they are a widely distributed group, as the following maps showing the distribution of the three species used in this study show.

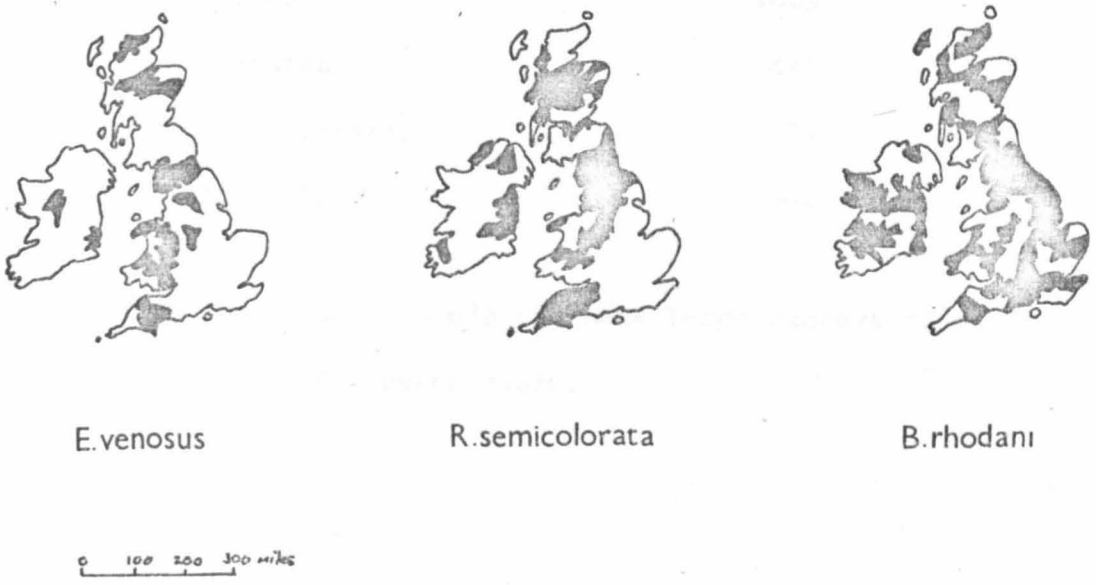


Fig.10 Distribution of mayfly species used in this study

There is also a certain amount of data which shows that where mayflies are found in a river or pond, they form a sizeable proportion of the biomass found within that particular ecosystem. The following is an extract from a table showing the numbers of animals, of different groups, found in a fairly typical habitat (Fordwood Beck).

TABLE 7 Animals of different groups found in Fordwood Beck

(For complete table see MACAN,T.T. 'Freshwater Ecology', pp. 17-19)

Taxonomic group	Number of animals per square metre
Platyhelminthes	48
Molluscs	42
Crustaceans	1432
Mayflies	1809
Stoneflies	445
Beetle (larvae)	17
Caddis fly	304

In the case of ponds the same large numbers of mayfly can be shown, as in the following table.

TABLE 8 Numbers of animals in Hudson's tarn living among growths of Carex and Littorella. (See MACAN,T.T. 'Freshwater Ecology' for full table.)

Taxonomic group	Carex	Littorella
Annelid worms	54	-
Molluscs	45	-
Crustaceans	18	-
Stoneflies	-	-
Mayflies	10,152	4158
Dragonflies	2862	1518
Caddisflies	162	144
Beetle (larvae)	-	19
Arachnids	216	79

The mayfly can, therefore, be seen to be a widely distributed insect, and one which occurs in large numbers within ecosystems. These are two qualities which make the aquatic stages of this insect good subjects for study. They are comparatively easy to collect in sufficient numbers and their wide distribution could also make them suitable types for use as indicator species. Also they are of some commercial importance, since they have been found to be among the species which form the principle day foods of the Trout (ELLIOTT, 1967).

Life Histories

Generally, the Ephemeroptera show three types of Life History (LANGFORD, 1971) which can be summarized as follows:

1. Nymphs that hatch in late summer or early autumn and grow to maturity as the temperature falls. These can be termed winter growers and R. semicolorata fits best into this category.

2. Nymphs that hatch in spring or early summer and grow to maturity during the warmest part of the year. These are therefore called summer growers and E. venosus is an example of this type, although it can also have a slow-growing winter generation (RAWLINSON, 1939).

3. Nymphs that are present all the year round in various stages of development, though growth is slow or almost stopped at certain times of the year. These species may have two generations every year or a prolonged emergence period which can stretch from April/May to September/October. B. rhodani is an example of this type and has a main emergence period from May to early June.

The role which temperature plays in the several life history phases is not yet all that well understood, but each species seems to be tolerant of a fairly wide range of temperature. Ephemerella ignita, for instance, which appears only during spring and summer, shows that it is tolerant of a natural range of from 8°C to 22°C in the Severn (LANGFORD, 1971), and Heptagenia sulphurea can survive the whole temperature range down to 0°C when newly hatched. These tolerances may be explainable by the nymphs exhibiting some resting

phase or diapause until conditions are suitable. MACAN (1957) has suggested that R. semicolorata avoids a too-early emergence by what may be a diapause stage in the large nymphs. ELLIOTT (1967) has recorded emerging sub-imagines of B. rhodani as late as November on Dartmoor although none could be found on the Severn. Here, however, it could be lack of food in the winter that is the factor for arrested growth and final development.

The ability of species to tolerate a wide temperature range at different stages in their life history could be a very significant factor, since it has been suggested that this ability is sufficient to enable the nymphs to withstand and survive even abnormal temperature conditions provided that these are not sustained. Also, a resting or diapase stage may have a significant effect on the apparent effect of a toxicant on test animals especially at the extremes of temperature ranges.

Activity

It has been found that there is a strong interrelation between the oxygen consumption of the nymph of Cloen dipterum (L) and its motor activity (NAGELL, 1973). Here variation of oxygen consumption at certain dissolved oxygen concentrations is explained by a variation in motor activity. Since oxygen consumption could have some effect on toxicity in terms of a general slowing down of metabolic rate, some information regarding normal activity patterns of some species of nymph would be useful.

ELLIOTT (1968) made a study of the daily activity patterns of

several Mayfly nymphs including those used in this investigation. He found that the nymphs of all the species that he studied showed constant patterns of activity under conditions of natural illumination, fairly constant temperature, oxygen consumption and flow rate. Activity was found to be greatest at night. B.rhodani was found to be generally the most active of the three species with R. semicolorata being the least active. Also, ELLIOTT found that continuous light or darkness did not change the activity pattern from that exhibited in natural illumination, although he did find the nymphs to be negatively phototaxic and in flowing water all the nymphs were strongly positively thigmotaxic. It is interesting that he found that in all except one of the nymph species the daily activity patterns changed markedly when the flow of water ceased. In still water he found that there were alternating periods of high and low activity throughout a period of 24 hours. B.rhodani showed a nocturnal periodicity in both drift rate and activity. The general conclusion arrived at by ELLIOTT in this study was that periodicity is probably controlled by light intensity and an endogenous rhythm. In normal conditions, however, (in terms of water current) mayfly nymphs show a diurnal rhythm which, once established, is not affected by either light or temperature. It has been suggested that this rhythm is developed very early in life (HARKER, 1953). Since such rhythms are more than likely to some extent inherent there may be a tendency for each species in a stream to develop its own rhythm within a specific microhabitat. This is due to the fact that the chances of the Mayfly fauna of one stream

mating with that of another are limited, due mainly to the short life of the sexually mature fly.

Although light may not have any extensive effect on the general diurnal rhythm of Mayflies there is evidence that it exerts an effect on orientation within these broad patterns of activity (HUGHES, 1966). Working with the nymph of Baetis Harrisoni, HUGHES found that there is a dorsal light response which orientates the animal in such a way that light is kept perpendicular to both the long and transverse axes of the body. This response maintains, or at least helps to maintain, the animal's normal dorso-ventral orientation in its natural environment. When there is no overhead light source disorientation occurs and this leads to an inability of nymphs to land effectively on the substrate. This observation could explain the phenomenon of drift in running waters at night. Also, there is a greater degree of activity at night due to periodicity which would also contribute greatly to factors causing more drift in the dark. HUGHES (1966) has carried out further work in which he has shown that the response of nymphs to various properties of light are important factors contributing to the nymph's selection and maintenance of their respective microhabitats.

* * * * *

The interrelationship between nymphs and habitat is a complex one, so that it is unlikely that all the factors which should be taken into consideration when carrying out laboratory studies are.

Nevertheless, it is of some importance to attempt to come as close as possible to natural conditions in the laboratory if the results obtained here are to be directly applied to the natural situation. If, for some factors, this is not practically possible then these should at least be borne in mind when the time comes to interpret results.

Chapter 4

THE TOXICITY TESTS, AND METHODS OF DATA PROCESSING

INTRODUCTION

As a consequence of the time which toxicity tests require this section forms the bulk of the experiments carried out in this investigation. The pattern they have followed is that which seems now to have become a fairly standard and familiar one to those who have worked in this field and it does not seem very worthwhile to reiterate the theoretical concepts behind this general pattern, since this has been covered in an earlier chapter. Points worth mentioning and explaining will be dealt with as they occur in the description of the tests themselves. It is hoped that some clarity will be gained as a result of the bulk, which is always the result of repetition, being lost.

The general aim of this long series of toxicity tests has been simply to gain data to which can be applied the techniques of processing leading to the production of Tlm curves and probit analyses. This data has been obtained from toxicity tests carried out with various complicating factors in an attempt to obtain a basic profile of the animal's reactions when subjected to various concentrations in different conditions. All these tests have been carried out in the laboratory and are therefore subject to certain limits of interpretation if the results obtained from them are ever used, or even viewed with the intention of imparting to them some practical application, such applications lying outside the substance of the actual results themselves as they stand.

The scheme or programme which the toxicity tests followed is summed up in the following table.

TABLE Scheme of toxicity tests
(All concentrations unless otherwise stated are expressed in mg/l $CuSO_4$)

Series A	Effect of high concentrations (10-200 mg/l)
Series AA	Effect of low concentrations (10-1 mg/l)
Series B	Comparison of effects of other salt of copper
Series C	Effect of temperature on toxicity
Series D	Effect of pH on toxicity
Series E	Effect of hardness on toxicity
Series F	Investigation of possible delayed action and sub-lethal concentrations

The experimental set-up for all these tests was the same throughout and will be described now.

The tests were carried out in a perspex 'race-track' channel which was provided with an electrically driven paddle-wheel so that a unidirectional current of water could be maintained throughout the test period. Providing this current is of some importance, since still water might possibly interfere with the respiration of the animals. The reasons for this have already been explained.

The race track was provided with entry and exit ducts so that a continually fresh supply of test solution could be provided. This was fed into the channels by means of a peristaltic pump which conveyed the test solution from a 20-litre bottle to the channel.

In addition, the channel was also supplied with a water heater controlled by a mercury/toluene thermostat. A diagrammatic representation of this set-up is shown in Fig. II As can be seen, a duplicate race-track to that described above was used for a parallel series of control runs. Both race-track channels were fitted with PVC gauze screens so that the animals could be confined to one area of the track. This was thought necessary to avoid loss of animals through the overflow outlet and to prevent possible damage of animals by the paddle wheel. Also it made observations and counts much easier.

The test solutions were made up in distilled water to which was added essential ions. The chemical make-up of these solutions was modified from that recommended by the Report of the Technical Committee on Fish Toxicity Tests published by the Ministry of Housing and Local Government in 1969. This recommends the addition of calcium chloride, sodium chloride, sodium nitrate, magnesium sulphate, sodium sulphate and sodium bicarbonate. In the test solutions used in this investigation the sodium bicarbonate was omitted since the bicarbonate radicle would have combined with the copper to give an insoluble copper compound which would be precipitated. Stock solutions containing all the chemicals listed above except the last were therefore made up. In all cases except for Series E test waters were made up with the following specifications.

FIG 11 CONTINUOUS FLOW APPARATUS FOR TOXICITY TESTS

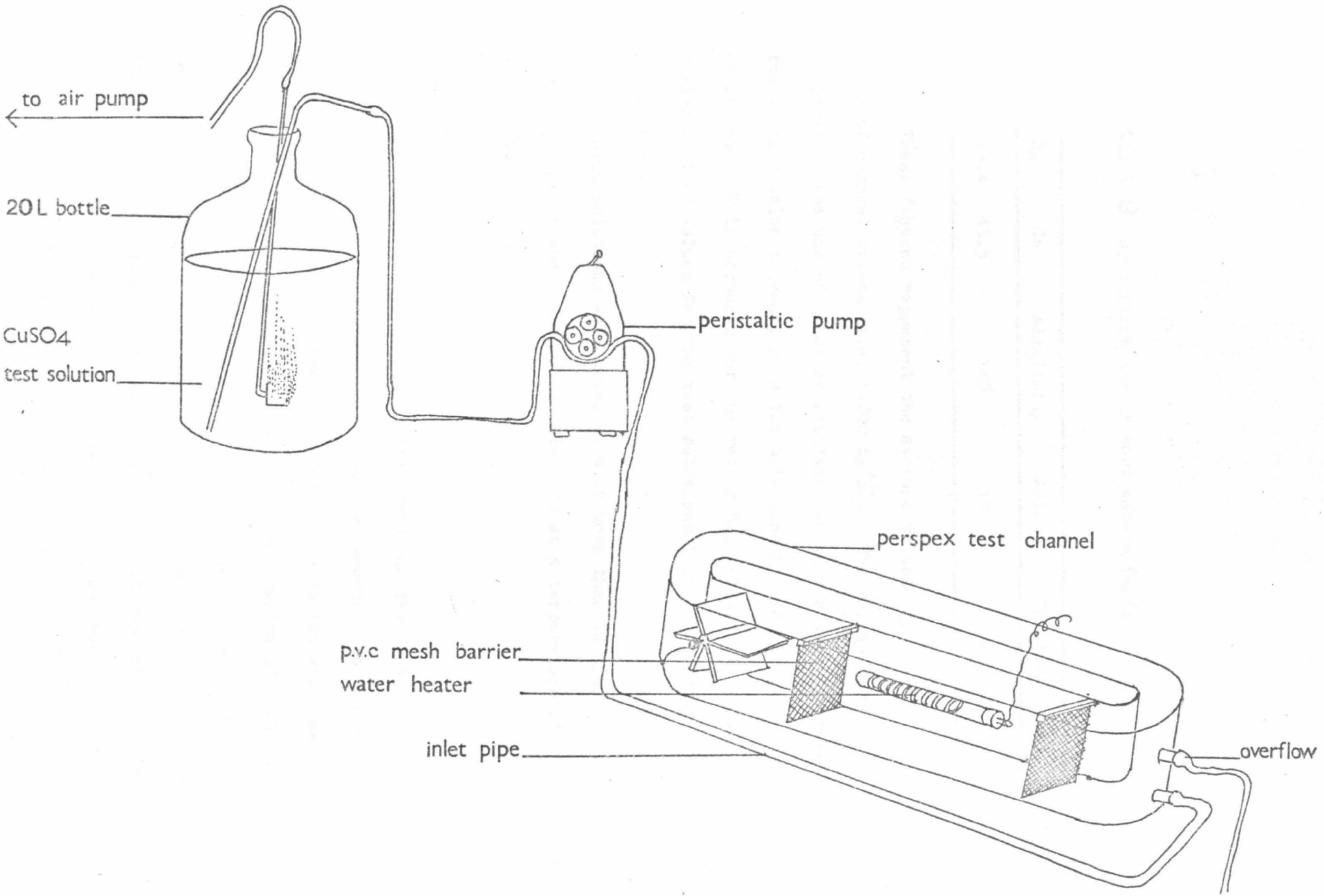


TABLE 9 Specifications of test waters (mg/l as $CaCO_3$)

Mg	Ca	Alkalinity	Acidity	Total hardness
19.4	45.5	140	10.3	64.9

These figures represent the average values for the entire range of concentrations (i.e. 1-200 mg/l). The values were achieved by the use of a buffer solution, which was used to prevent the acidity value increasing in the more concentrated copper solutions. This accounts for the rather high alkalinity value. A fuller list of values for the test solutions 1-200 mg/l.$CuSO_4$ is given later.

These solutions were never made up more than 12 hours before they were needed and stored in a cold room at a temperature of about $5^{o}C$.

Procedure

The method used in the toxicity tests was the same throughout the entire series. The test solutions were poured into their respective race-tracks and brought up (or down) to the experimental temperature. The animals were usually given a period of acclimatization by placing them in a control test solution for a period of not less than six hours. They were not starved during acclimatization and were provided with a good supply of air as it was not possible to keep them in moving water for this period. It was hoped that this procedure would avoid shock effect, especially in the case of tests

carried out at higher temperatures and extremes of pH. In relation to the animals it might be a good thing to digress slightly at this point to go into a little more detail regarding their treatment.

Most of the animals used for this work were obtained from the River Coquet at a site about 2½ miles south of Rothbury. They were collected from the river bed by means of a fine mesh hand net which was used to dredge small stones from the bed. These were transferred to an enamel tray and the nymphs collected with a glass bulb pipette. At this stage animals which were seen to be damaged were discarded. The nymphs collected in this way were placed in a glass bottle of river water for transportation to the laboratory. The collecting period was usually 1 - 1½ hours and the numbers of nymphs collected in this time varied considerably. Normally the Coquet at the collecting site is about 18-24 inches deep and in these conditions it is possible to collect 80-150 nymphs. However, the Coquet easily floods and in flood it is difficult to collect, since many animals will have been swept downstream.

In the laboratory the nymphs were kept in a large perspex race-track supplied with a variable speed paddle wheel, aerator and water cooler. The water here was a mixture of river and tap water, and by providing stones and vegetation it was possible to keep nymphs alive and so build up a stock for use in the experiments. It was found that loss of animals due to mortality and metamorphosis was reduced if the water temperature was not allowed to rise above 10°C.

Animals required for toxicity tests were collected from this artificial stream with a glass bulb pipette and transferred to a

beaker for the acclimatization period. For the tests 20 animals were used, 10 for the test channel and 10 for the control. Prior to transferring the nymphs to the actual test channels they were treated as described above. The nymphs were then placed into the channels and the experiment was started by switching on the peristaltic pumps, so that a flow of fresh solution began entering the channels and starting the paddlewheels. The water heaters, being thermostatically controlled and being already set at the required temperature, needed no further attention.

Observations of survival were made every half hour throughout the duration of the experiment for all series except AA and parts of F. In Series AA observations were made every 12 hours since the time span here was much greater than that of the other series. In all series (except AA) the temperature and dissolved oxygen concentration (and pH in the case of Series D) were monitored every three hours by means of an ordinary centigrade mercury thermometer for temperature and the use of the Winkler method for dissolved oxygen.

Criteria for death

One may ask how it was decided at what point test animals could be declared to be dead. For the purposes of this study an animal was considered dead when all movement had ceased for a period of not less than one hour, i.e. if no movement could be observed in a nymph during two observation periods. A small glass rod was used each time to try to elicit a response to tactile stimulation.

This was the ultimate and absolute criterion which was used

but there are several stages which the nymphs can visibly be seen to go through from the time they are placed in the toxic solution to the time of death. The remarks which are entered in the data sheets (see appendix) for the toxicity tests are based on these stages, so it would be well to describe them here with special emphasis on the way they are used to describe in a few words the states of the test nymphs as the experiment proceeds.

1. 'Activity' or 'active'

These words are used to signify the normal state of the animals when they are first placed in the solution unless they are accompanied by a qualification. Here the nymphs can be seen swimming and clinging on to the sides of the channels and retaining meshes with their bodies arched characteristically. Also they respond very rapidly to tactile stimuli.

2. 'Animals on backs'

This seems to be the first real indication that something is wrong. Here a nymph can be seen on its back at the bottom of the channel. If touched with a glass rod it will either right itself and swim away or will remain on its back but still move its legs energetically. An entry of an animal being on its back was only made on the data sheets if it failed to swim after a tactile stimulus.

Often animals would remain on their backs with their legs making energetic movements without the need of any stimulation. Here the bodies would still be well arched backwards. This arching of the

body and the general way in which the nymph 'held' itself became an important diagnostic feature in determining the state of a particular nymph.

3. 'Animals on backs and weak'

Here the nymphs stay on their backs for long periods of time and fail to respond to repeated tactile stimuli. The legs still move, but only sporadically. The gills are still moving, but only intermittently. The bodies are beginning to lose the arching.

4. 'Animals on backs and very weak' or 'very weak'

Here no movement of the legs can be seen at all, even when mechanically stimulated. Only the gills can be seen to move, these movements being weak and eratic. The legs are becoming withdrawn and held close to the body. No movement can be observed of the antennae.

5. Death

The antennae, gills and legs show no movement and cannot be made to move by tactile stimuli. The legs are completely bunched, close to the ventral surface of the body. The body itself has assumed a characteristic 'slouch' in which there is no trace of a dorsal arching, this has been replaced by a ventral bending of the body along its longitudinal axis.

What have been described here are the main terms used in describing the nymphs during the course of the toxicity tests. Other terms which are fairly self-explanatory are also used from time to time.

THE TESTS

Series A

This series consisted of a set of toxicity tests designed to give a basic profile for copper toxicity over a range of concentrations. As such, it must be considered a preliminary series. The concentrations used were 10, 20, 50, 100 and 200 mg/litre of copper sulphate. These were made up in distilled water containing the ions as described above. In the higher concentrations a buffer was used to prevent the resulting acidity producing too low a pH value. This precaution was also carried out in subsequent tests where high concentrations of copper sulphate were used.

All three species of nymph were used in this series and as far as possible precautions were taken to ensure that the treatment given to all three species was the same. In all cases 10 nymphs were used in each test, with another 10 in the control channel.

High concentrations were used in this series because it was found that, given the right environmental conditions these animals were very resistant to abnormal copper amounts in the water. Therefore, early in this investigation it was decided to have a separate section dealing with the lower concentrations to try to establish thresholds of toxicity.

Series AA

The object of this series was to try to find threshold concentrations at which copper would start to exert a chronic toxicity effect on the nymphs. The concentrations used were 10, 7, 5, 3 and

1 mg/l $CuSO_4$. Observations were made every twelve hours, since most of these tests spanned several days to two weeks and counts every half-hour would not have been a practical possibility. As in Series A, dissolved oxygen and temperature were regularly monitored, in this case at 12-hour intervals.

Since these tests were so long it was necessary to provide the animals with vegetation so that they could feed normally. The vegetation supplied was grown in a specially prepared copper-free medium to ensure that the only source of copper came from that in the test solutions. Also the plants were killed by boiling in distilled water before placing them in the experimental channels to prevent any copper uptake by them. This was probably an extreme precaution since the amount of plant material used compared to the total volume of test solution was very small indeed.

The introduction of vegetation introduces a small unknown factor in that feeding nymphs will also be taking in some water through the mouth. If this is the method by which copper enters the body from the external medium then differential rates of feeding in individual nymphs may introduce a non-measurable factor, albeit a very small one. Whether this makes any difference in the long run is a debatable point, but it seems to me that active or incidental drinking in of the medium could make a not negligible difference to the apparent toxicity of a substance as measured by normal methods. To test this out two runs were carried out, with a fairly high concentration to give a quick result, in which plant material was supplied in one channel but not in the other. The result, using

this crude technique, showed no significant difference in the survival curves of the two sets of nymphs. More sophisticated experiments into this detail could, however, be carried out to resolve the problem fully.

Series B

Here a concentration range similar to that used in Series A was employed, but in this case different copper salts were used. These were copper nitrate and copper chloride. It was thought a good idea to do this to check any possible effect that the sulphate radical could be exerting in the overall toxicity. If copper chloride and nitrate gave the same or at least very similar results as each other and copper sulphate it would be reasonable to assume that these radicals did not contribute to the toxic action of the salts, i.e. it would be the copper that was responsible for toxicity. Also in this series a number of runs using magnesium sulphate were made to test for any effect of the sulphate radical.

The concentrations used were fairly high and adjusted so that the actual copper content in each case was the same, i.e.

84.7 mg/l cupric chloride ≡ 40 mg/l copper

152.1 mg/l copper nitrate ≡ 40 mg/l copper

1006 mg/l copper sulphate ≡ 40 mg/l copper

This series was restricted to one run at this concentration for each species of nymph. The results were subjected to a probit analysis.

Series C

This series was carried out in order to determine the effect of temperature on the toxicity of copper, i.e. once survival curves had been established at a given temperature I wanted to find out how these curves were affected by changes in temperature.

All the series described so far were carried out at a temperature of 10°C, this being taken as a reasonable temperature to use as 'normal'. In nature, however, there are sometimes drastic temperature changes and it is important that something is known about the effects these changes are going to have on the action of possible pollutants in natural waters. Also, industrial processes often require cooling water which is extracted from a river and then returned to it at a much higher temperature, thus increasing the overall temperature of the water for quite considerable distances. Calculated safe levels for a certain effluent become, as a consequence, meaningless unless the effect of temperature on its action is investigated also.

To give a very general idea of natural temperature variations in water bodies a very brief summary of thermal conditions in different types of lakes is here included (based on YOSHIMURA,1936a).

1. Tropical lakes

   These have high surface temperatures of 20 to 30°C. They have a small annual amplitude of variation and a small thermal gradient.

2. Sub-tropical lakes

   These have a surface temperature never below 4°C. The annual variation is large and thermal gradients are large.

3. Temperate lakes

These have a surface temperature above 4°C in the summer and below 4°C in the winter. Thermal gradients and seasonal variations are large.

4. Sub-polar lakes

These have surface temperatures of above 4°C only for a short time in summer. The thermal gradient is small.

5. Polar lakes

These have surface temperatures always below 4°C.

Taking a global view, therefore, it can be seen that temperature variations occur naturally from one part of the earth to another, without the intervention of man. Generally temperatures vary from 30°C to below 4°C and often there are rapid changes in temperature within individual bodies of water.

In rivers there are also climatic variations in water temperatures, as well as marked seasonal variations within each river. Added to this there must be the consideration that the temperature of the water in a river will vary according to its different zones. Therefore, headstreams, which are likely to be torrential, will generally have comparatively low temperatures with little seasonal variation, whereas lower, broader and slower reaches will be warmer and subject to a greater seasonal variation.

Taking these several considerations into account it would seem that some knowledge of the way a toxic effluent is likely to behave under different temperature regimes is essential before any widespread application of that toxicant which, as in the case of

copper, may be quite deliberate, i.e. as an antifouling agent, or as an incidental part of some industrial processes where an unwanted by-product is ejected into water systems.

This series, which was a large one, was therefore planned to give some basic information regarding the effect of different temperatures on the toxicity of copper. The temperature range used was 5, 10, 15, 20 and 25°C. Each of these temperatures was used in conjunction with the range of concentrations used in Series A. Copper sulphate was used and the procedure was identical counts of survival being taken every half-hour. The required temperatures were achieved by use of the submerged water heaters described above. An acclimatization period at the test temperature was allowed for all three species.

The dissolved oxygen was kept as constant as possible by regulating the water aerator, but here there is some variation throughout the series in that the test solutions at the lower end of the temperature range do have more dissolved oxygen than those at the higher end (see data sheets). Again, dissolved oxygen and temperature were monitored every three hours.

Series D

This series of runs deals with the effect of varying pH on the toxic effect of copper. pH, like temperature, can vary because of causes both natural and unnatural. Variation due to natural factors, e.g. local geology through which a river runs or on which a lake lies are not normally very great, but they have been large

enough to make it possible to classify rivers purely using pH as a criterion of classification. Thus, in 1961 HARRISON and AGNEW classified three types of rivers according to pH, as follows:

1. pH 5.0 to 5.9
2. pH 6.0 to 6.9
3. pH 7.0 to 8.5

The pH of a river or lake can have far-reaching effects on the type of fauna and flora that will be found living in them, and many species of animals or plants may be excluded because the pH is too high or too low. Although the pH variation due to natural causes may be small between one river and another or one lake and another, and although fluctuations within each river or lake may be negligible, by-products of industrial processes could change the pH of a water system drastically. As with temperature, therefore, pH must be considered as a possible complicating factor as it could affect the toxicity of copper. It is important, therefore, that some basic knowledge of what this effect might be is attained.

In this series four pH values were used, 3, 4, 5 and 7. pH values above 7 were not used since above this value copper is precipitated out of solution and therefore would be effectively removed as a potential toxicant.

The required pH values were obtained by the addition of previously calculated amounts of sulphuric acid. For this series the controls consisted of test water made up to the specifications given above, but at the pH of the test, i.e. the test and control solutions were identical except, of course, for the absence of

copper in the control water. This was done in order to determine the effect of pH alone at the same time as determining this superimposed on copper of different concentrations. These concentrations were 10, 50 and 200 mg/l $CuSO_4$. The runs were, therefore, as follows:

pH 7, 5, 4 and 3 with 10 mg/l $CuSO_4$

pH 7, 5, 4 and 3 with 50 mg/l $CuSO_4$

pH 7, 5, 4 and 3 with 200 mg/l $CuSO_4$

The temperature used for this series was 10°C and the pH was monitored every three hours using a portable pH meter. Samples of water were taken from the overflows of both test and control channel and tested with the meter.

Series E

This series deals with hardness as a complicating factor. This factor has also been used in the past as a basis for the classification of water systems. Hardness is, therefore, also a factor which fluctuates enough in nature to make it possible to differentiate between bodies of water and river systems. In 1933 BUTCHER described four types of river using hardness expressed as ppm of calcium carbonate. These were:

1. No Ca (highly acid) less than 2.0ppm calcium carbonate
2. Slightly calcareous. 2.0 - 10.0ppm calcium carbonate
3. Moderately calcareous. 10.0 - 20.0ppm calcium carbonate
4. Highly calcareous. More than 20ppm calcium carbonate

Another example of such a classification is that of OHLE(1932):

1. Ca poor. 0 - 14 mg/l CaO
2. Intermediate. 14 - 36 mg/l CaO
3. Ca rich. 36 + mg/l CaO

and in 1955 DITTMAR used the association between calcium and magnesium and produced a classification based on two types of river:

1. Gammarus. Ca/Mg ratio greater than 2/1
2. Niphargus. Ca/Mg ratio less than 2/1

Hardness, being variable, could therefore be a factor that affects the toxicity of a substance; in fact, it has been shown that a high calcium content reduces the toxicity of heavy metals to organisms (JONES, 1938; HERBERT and WAKEFORD, 1962).

By altering the amounts of stock solution added to the test solutions it was possible to obtain the following range of hardnesses in the test and control waters:

	Total hardness mg/l $CaCO_3$	Calcium	Magnesium
1.	102.6	79.6	23.0
2.	64.5	45.5	19.5
3.	38.8	27.3	11.5
4.	10.3	7.9	2.4
5.	6.5	4.5	2.0

As with Series D, each of these hardnesses was used with copper sulphate concentrations of 10, 50 and 200 mg/l for all three species of nymph. The hardness of the solutions was determined by the EDTA titrimetric method for calcium and the gravimetric method for magnesium. Both of these methods are described in Standard

Methods for the Examination of Water and Wastewater, 11th Edition, 1960.

Series F

All the series so far described have dealt with essentially chronic toxicity in a variety of conditions, i.e. the experiments have been continued (in most cases) until all the animals in the test channels have been killed by the copper in solution. Series AA dealt with the threshold concentrations at which copper just began to exert a toxic effect. This series (F) is an attempt at obtaining information regarding sub-lethal concentrations not in relation to chronix toxicity but in relation to recovery of the animals after they have been exposed to toxic solution for certain lengths of time, removed from these, and placed in a non-toxic solution. Here the aim of the experiments was to place the animals, as before, in known toxic solutions for varying periods of time and then to measure the recovery rate.

The same range of concentrations was used as in Series A and the experimental set-up and procedure was identical, except for the fact that in this case the animals were fed during the course of the experiment. The exposure periods varied according to the strength of solution. A table summarizing this is given below.

TABLE 10 Summary of exposure times for Series F.

Conc. mg/l $CuSO_4$	Exposure times in hours					
10	2	4	6	8	10	12
20	2	4	6	-	-	-
50	1	2	3	4	5	-
100	1	2	3	4	-	-
200	1	2	3	4	-	-

The aim in choosing these times has been to give a range of times, the maximum of which is within the time taken for copper to begin having a toxic action (as measured by mortality), this information being obtained from the results of the toxicity tests constituting Series A.

After each of these time periods expired the animals were removed carefully and placed in a beaker containing well aerated test solution (copper free) while the perspex channel was drained and thoroughly washed out several times with distilled water and then filled again, this time with copper-free test solution. The animals were then replaced and the experiment continued, keeping to the original timing for that run. A control perspex channel with copper-free solution was also maintained throughout the runs, as in all the other series so far described. Observations were made every half-hour and the dissolved oxygen and temperature ($10^{o}C$) were monitored every three hours, up to the time when the animals were removed from the toxic solution.

For this series only one of the species was used, B.rhodani.

After each of the toxicity tests described above the perspex channels were carefully drained of the copper solution and given several washings with distilled water. If for some reason another test was not to be carried out for several days, the channel was filled with distilled water for that period. All tubes, rubber and glass were disconnected after each run and carefully washed with distilled water. The 20L stock bottles were also cleaned out between runs.

The waste copper solution that left the perspex channel by way of the overflow outlet (see Fig.11) was discarded. It was suggested that an analysis could be carried out of this to determine the amount of copper that had, in fact, been taken up by the nymphs during the course of the experiment. This was not done, however, since these animals were in a continuously replenishing solution and during the course of some runs as much as 60 litres of solution would have passed through the channels. The size of the nymphs is such that so little copper would be taken up from 60 litres that it seemed optimistic, to say the least, that this minute quantity would be detected.

For each species used it was attempted to use nymphs of a more or less uniform size throughout the series A to F. In this way the data obtained from one run would be more comparable with that of another using the same species. No attempt was made to obtain a uniformity of size or weight embracing all three species, since the difference in size and weight could be a factor contributing towards any differences in the response of the respective species. Any

attempt at uniformity between different species would have eliminated this difference which would be present in nature.

All the survival and mortality data from these toxicity tests was entered on sheets showing the time, survival of test and control animals, the dissolved oxygen and temperature, together with a column of brief descriptive remarks (see above).

PROCESSING OF TOXICITY TEST DATA

The data obtained from the series of toxicity tests A to F was handled mainly in two ways. It was used firstly to obtain Tlm survival curves, and secondly it was subjected to a probit analysis.

Tlm (median tolerance limit) is really synonymous with LC50 which stands for median lethal concentration, and expresses the concentration that will give a 50% mortality in a certain period of time for a given toxic substance. This time plotted against different concentrations gives a survival curve which is a basic profile of how animals will react to concentrations of different strengths.

In making a survival curve a first plot is constructed from the data. Here, concentration is plotted against survival (expressed as a percentage of the animals used in a test) at different points in time during the course of a toxicity test.

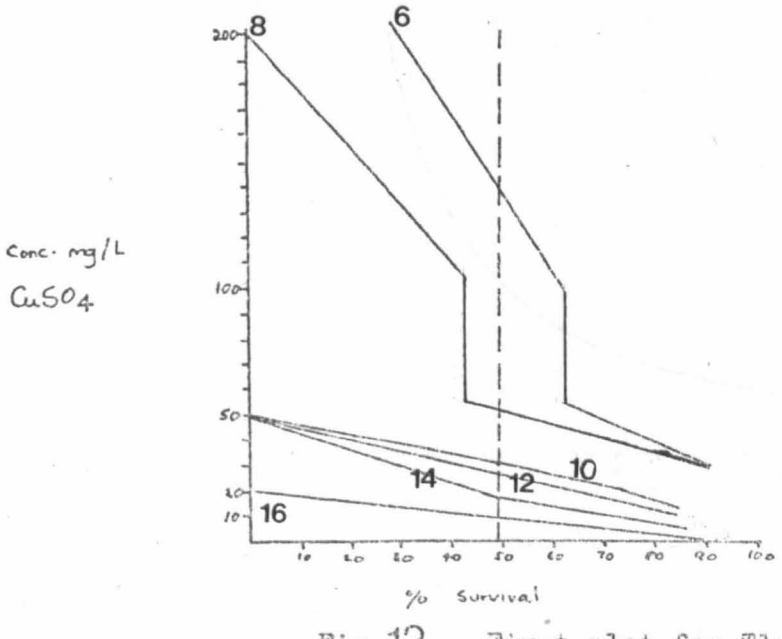


Fig. 12 First plot for Tlm survival curve

When this graph has been plotted a line is drawn through the different time curves, perpendicularly from the 50% survival point on the horizontal axis. At the points at which this 50% line transects the survival curves is read off a series of concentrations from the vertical axis. This new data is then used to construct the second plot which is the survival curve. This is done by plotting the range of concentrations obtained above against time.

This is preferable to using a simple mean for survival as it does, to a certain extent, eliminate extremes of response.

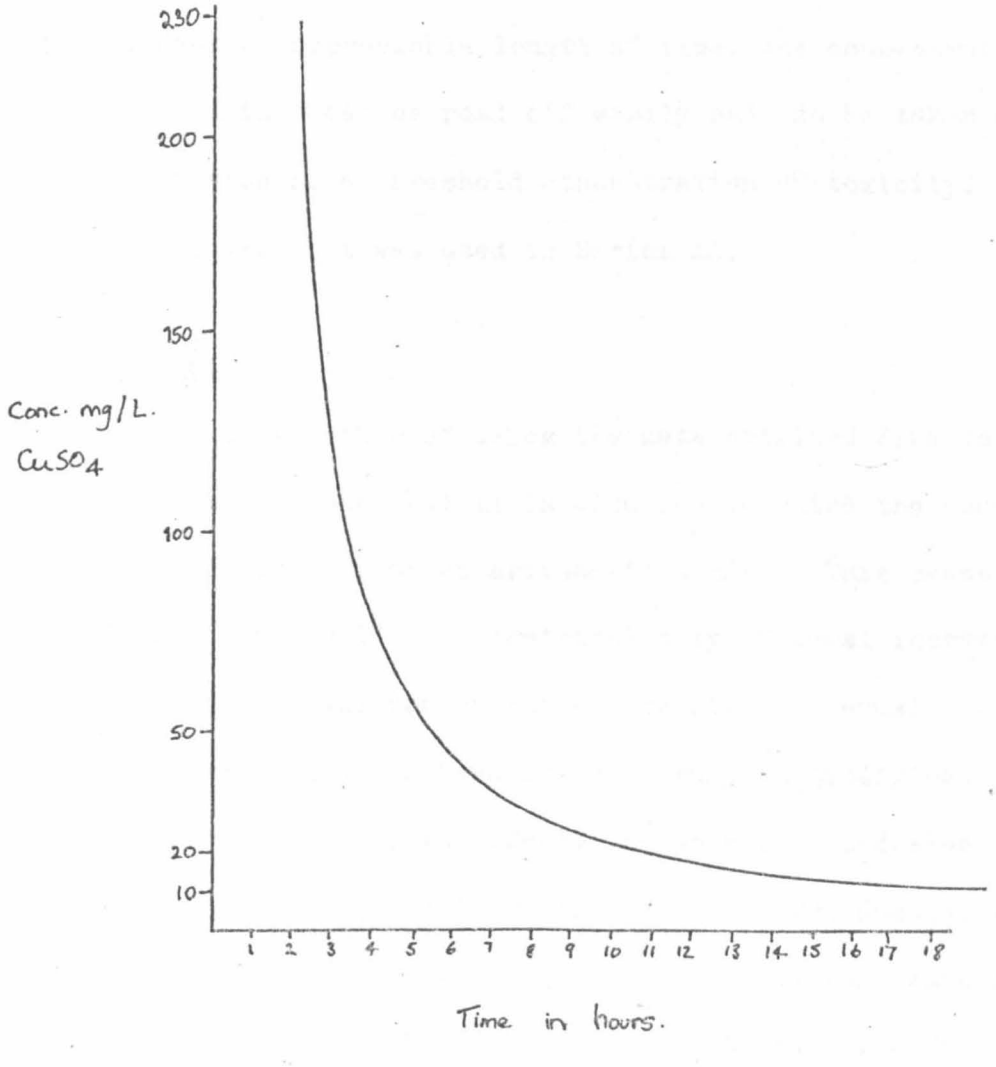


Fig 13 Second plot, the survival curve

These extremes could be due to certain animals being damaged in some way that would lessen their tolerance to copper, or to animals being, for some reason, naturally more resistant to copper than most. Using Tlm does help to level out such extremes of response, whatever their cause, and give what can be taken as a general theoretical response for a particular species. The curve also gives a very good visual representation of the extent of toxicity at different concentrations because, as a concentration becomes less toxic, the time at which 50% of the animals will survive increases and the curve becomes more horizontal. Once this horizontal part is arrived at and maintained over an appreciable length of time, the concentration at which it is maintained can be read off easily and can be taken as a representation of a threshold concentration of toxicity. It was this procedure that was used in Series AA.

Probit analysis

The above method of using the data obtained from toxicity tests is a very useful one, but it is also one in which the concentrations are plotted directly on an arithmetic scale. This means that the resulting curve could be symmetrical only if equal increments in concentration at all concentrations resulted in equal increments in lethal action. It has been noted in many physiological processes that equal increments, in effect, are only produced when the stimulus is increased by a constant proportion of a given dosage, rather than by a constant amount. It seems probable that this same rule might also hold true for toxicological processes, in which case dosage would have to be plotted in logarithmic terms to show a

uniform increase in kill. This is underlined by SPRAGUE (1969), who points out that if cumulated percentage mortality or survival from a toxicity test is plotted against exposure time, a skewed sigmoid curve usually results. This curve tails off more gradually towards longer survival times and, SPRAGUE points out, the skew apparently results from the logarithmic nature of biological time (GADDUM,1953; LECOMTE DU NOILY,1937). This skew can be eliminated by using a logarithmic scale on the time axis. As SPRAGUE explains, the sigmoid nature of the curve results from variable individual response causing the curve to resemble a cumulated normal curve, or a cumulated lognormal curve. This is usually straightened out by plotting percentage response (in terms of mortality or survival) on a probability (≡ probit) scale instead of an arithmetic. Probits, therefore, express mortality (or survival) in terms of standard deviations above and below the mean response, with the value 5.0 added to eliminate negative numbers (BLISS and CATTEL, 1943).

There are two more ways in which a probit analysis could be carried out. One of these is by the use of graph paper which has been ruled in such a way that a relationship involving the two functions, the cumulative curve (as ordinate) and logarithms (as abscissa), would plot as a straight line. The second method is to transform the data instead of the paper to appropriate units. The transformation of concentration is no problem, since one has only to make use of a table of easily accessible logarithms. For the percentage of mortality there was no equally simple and direct way out. For this reason BLISS (1934) derived a table of arbitrary

probability units which he termed 'probits'. It is this second method, using BLISS's probit units, that has been used in this study and applied to much of the data obtained in the above tests. As BLISS points out, this type of presentation of data has the following advantages.

1. It is a test of the theory of toxic action that:
   a) the variation in susceptibility among individuals is normal; and
   b) the effectiveness of the dose increases as its logarithm.

2. It gives a closer scrutiny of experimental techniques to determine if the organisms exposed to each concentration were truly equivalent, and if the amounts administered experimentally were uniformly proportional to the effective dosage over the entire range covered by the toxicity tests.

3. (Perhaps the most important) It shows up any change in the mode of lethal action with certain poisons over different sections of the dosage range (and in different chemical and physical conditions), indicated by an abrupt change in slope.

4. It is a simple method for expressing, in the slope of a straight line, the relative uniformity or diversity between individual species in their susceptibility to a poison.

Chapter 5

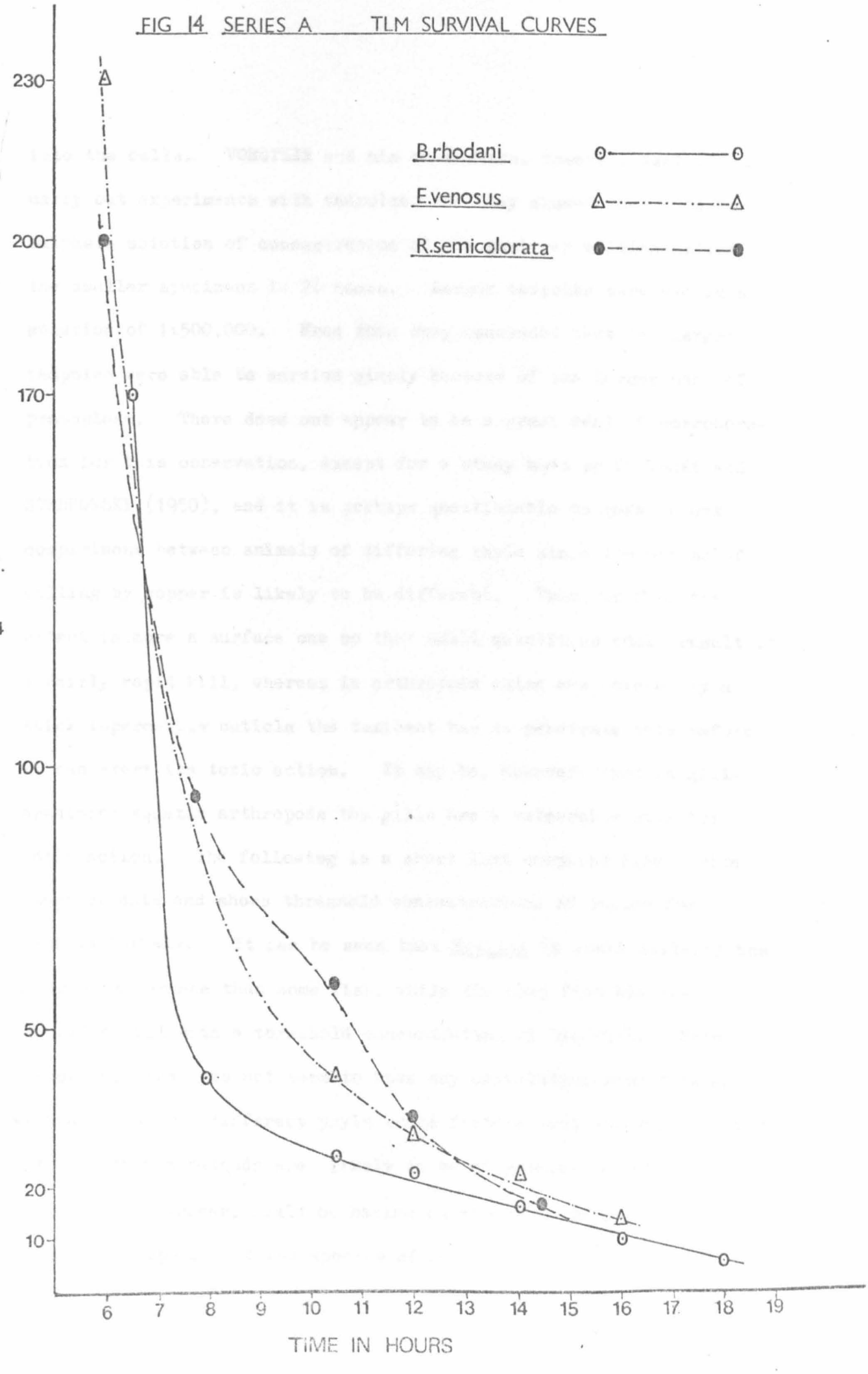
RESULTS OF TOXICITY TESTS

DISCUSSION OF RESULTS

SERIES A

Fig.14 shows the Tlm survival curves for the species B.rhodani, E.venosus and R.semicolorata. As can be seen, in all cases there is a marked toxic effect down to 10mg/l copper sulphate since there appears to be no tendency for the curves to flatten out and become horizontal towards the lower concentration end of the graphs. Also, according to this graph, B.rhodani seems to be the most susceptible of the three species, with E.venosus occupying a middle position. The explanation for this may lie in the fact that of the three species used, B.rhodani is the most active. It has the 'fishlike' body shape and will, in nature, be the species which will swim the most of the three. Thus, being the most active, it will also have a higher metabolic rate than either E.venosus or R.semicolorata, and therefore can be expected to take up copper and metabolise it faster. It is also possible that the fact that B.rhodani happens to be the smallest of the three nymphs contributes to its greater susceptibility. This question of size being a factor which contributes to the extent of toxic effect appears to have some support in the work of VOEGTLIN, JOHNSON and DYER (1925). Here it is argued that a thread of Spirogyra may weigh only a fraction of a milligram and on exposure to a large volume (100cc) of very dilute copper sulphate solution (1:10 million) enough metal ions are present to exert a toxic effect by chemical means. It is true that a thread of Spirogyra is not immediately comparable to mayfly nymphs, since very small organisms such as algae and bacteria have large surface areas and furnish ideal conditions for adsorption of the metal ions with subsequent uptake

FIG 14 SERIES A TLM SURVIVAL CURVES



into the cells. VOEGTLIN and his co-workers, however, went on to carry out experiments with tadpoles, and they showed that a copper sulphate solution of concentration of one part per million killed the smaller specimens in 2½ hours. Larger tadpoles survived in a solution of 1:500,000. From this they concluded that the larger tadpoles were able tc survive simply because of the larger mass of protoplasm. There does not appear to be a great deal of corroboration for this observation, except for a study made by PODUBSKY and STEDRONSKY (1950), and it is perhaps questionable to make direct comparisons between animals of differing phyla since the method of killing by copper is likely to be different. Thus, in fish the effect is more a surface one so that small quantities could result in a fairly rapid kill, whereas in arthropods which are covered by a thick impermeable cuticle the toxicant has to penetrate this before it can exert its toxic action. It may be, however, that in gill-breathing aquatic arthropods the gills are a vulnerable site for toxic action. The following is a short list compiled from a wide range of data and shows threshold concentrations of copper for various animals. It can be seen that Mytilus (a small mollusc) has a higher tolerance than some fish, while the cray fish has the highest of all with a threshold concentration of 3mg Cu/L. Here, therefore, size does not seem to have any correlation when dealing with animals from different phyla where factors such as body covering and respiration methods are likely to be of greater importance. The size factor, however, could be having an effect in determining the effects of copper to three species of animals from much smaller

taxonomic groups as in the case of the three mayfly species used here. What does seem fairly certain, however, is that all three are relatively resistant to copper, E.venosus being capable of surviving for a period of about six hours in a concentration of 230mg/l of copper sulphate, R.semicolorata and B.rhodani tolerating concentrations of 200 and 170mg/l copper sulphate respectively for six hours. These values are high, however, only with respect to the laboratory situation, since in nature six hours is not a long time.

TABLE 11 THRESHOLD CONCENTRATIONS FOR VARIOUS ANIMALS

Animal	Threshold concentration expressed as mgCu/L
Trout	0.09
Carp	0.22
Goldfish	0.33
Sunfish	0.89
Blackbass	1.32
Bluegill	0.53
Mytilus	0.55 (CLARKE,1947)
Crayfish	3.00 (HUBSCHMAN, 1967)
Daphnia	0.10 (BRINGMANN and KÜHN,1959)
Polycelis nigra	0.31 (JONES,1940)

Arthropods do in general seem to emerge as a rather resistant group, a quality which can perhaps be explained in termsof the possession of an outer covering which is very successful at keeping most things out.

SERIES AA (Threshold of toxicity)

Figs.15,16 and 17 show the T1m survival curves for concentrations ranging from 1 to about 10mg/l copper sulphate. As can be seen, at these lower concentrations the curves do reach horizontal positions and the concentrations corresponding to these parts of the curves have been taken as the thresholds of toxic effect for each species. These values are as follows:

TABLE 12 THRESHOLD CONCENTRATIONS FOR THE THREE SPECIES

Species	Conc. mg/l $CuSO_4$	Conc. mg/l Copper
R.semicolorata	1.9	0.76
E.venosus	1.7	0.68
B.rhodani	1.2	0.48

Thus the same order of resistance is exposed here as that shown by Series A, with B.rhodani being the first to be affected.

How do these values fit in with those which have already been arrived at for other organisms? Table 13 below shows some of these values with those of the mayfly species included.

The arthropods do seem to form a relatively resistant group but there is still not enough data available to make this more than a cautious generalization. The mayflies seem to occupy a fairly high position in terms of tolerance in respect to other organisms.

It must be said that these threshold concentrations should be interpreted as representing only a rough or approximate value. This is because for one thing, in biological systems there are rarely absolute or fixed values. The response of one animal compared to

FIG 15 SERIES AA THRESHOLD OF TOXICITY TLM PLOT R.semicolorata

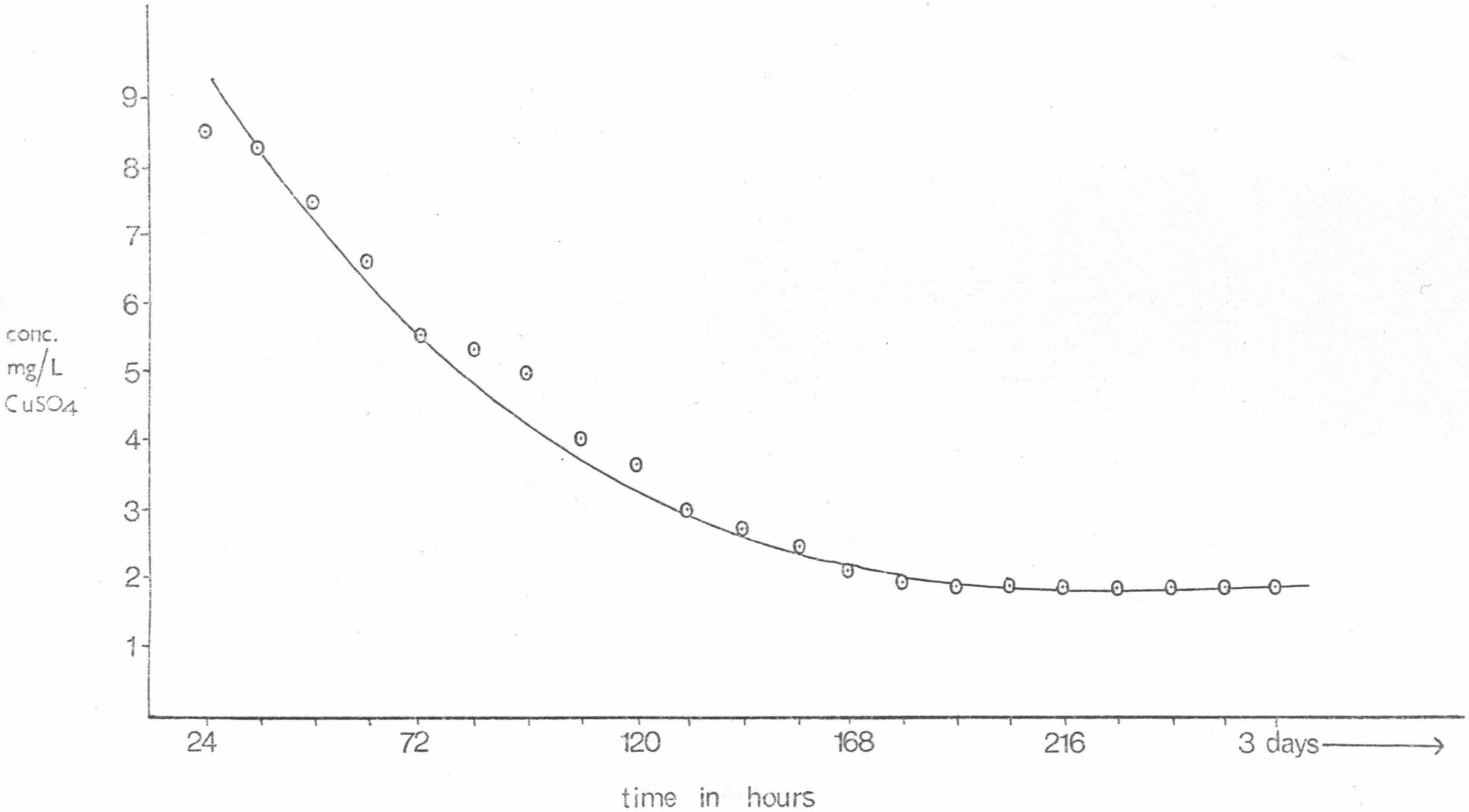


FIG16 SERIES AA THRESHOLD OF TOXICITY TLM PLOT E.venosus

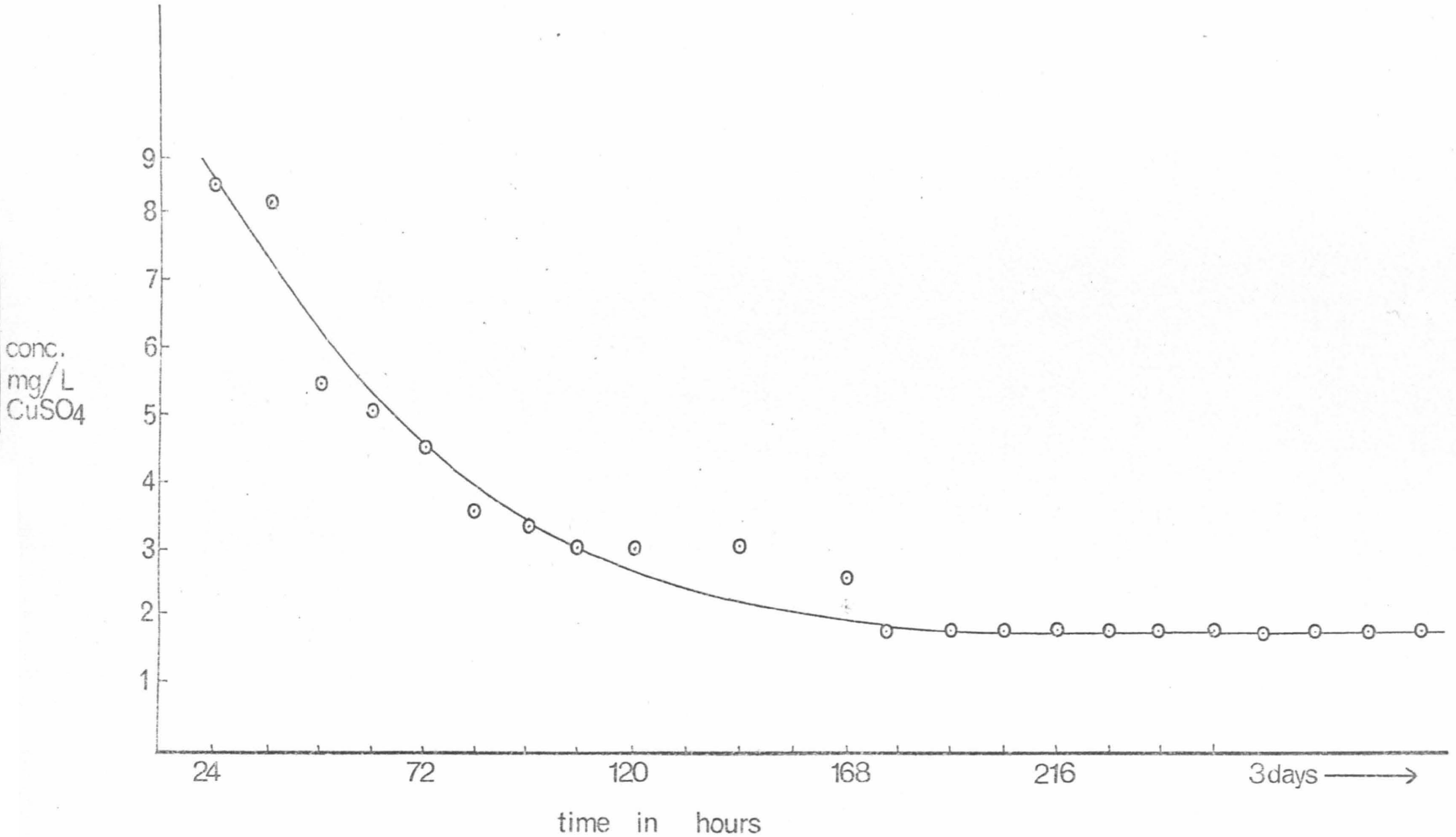
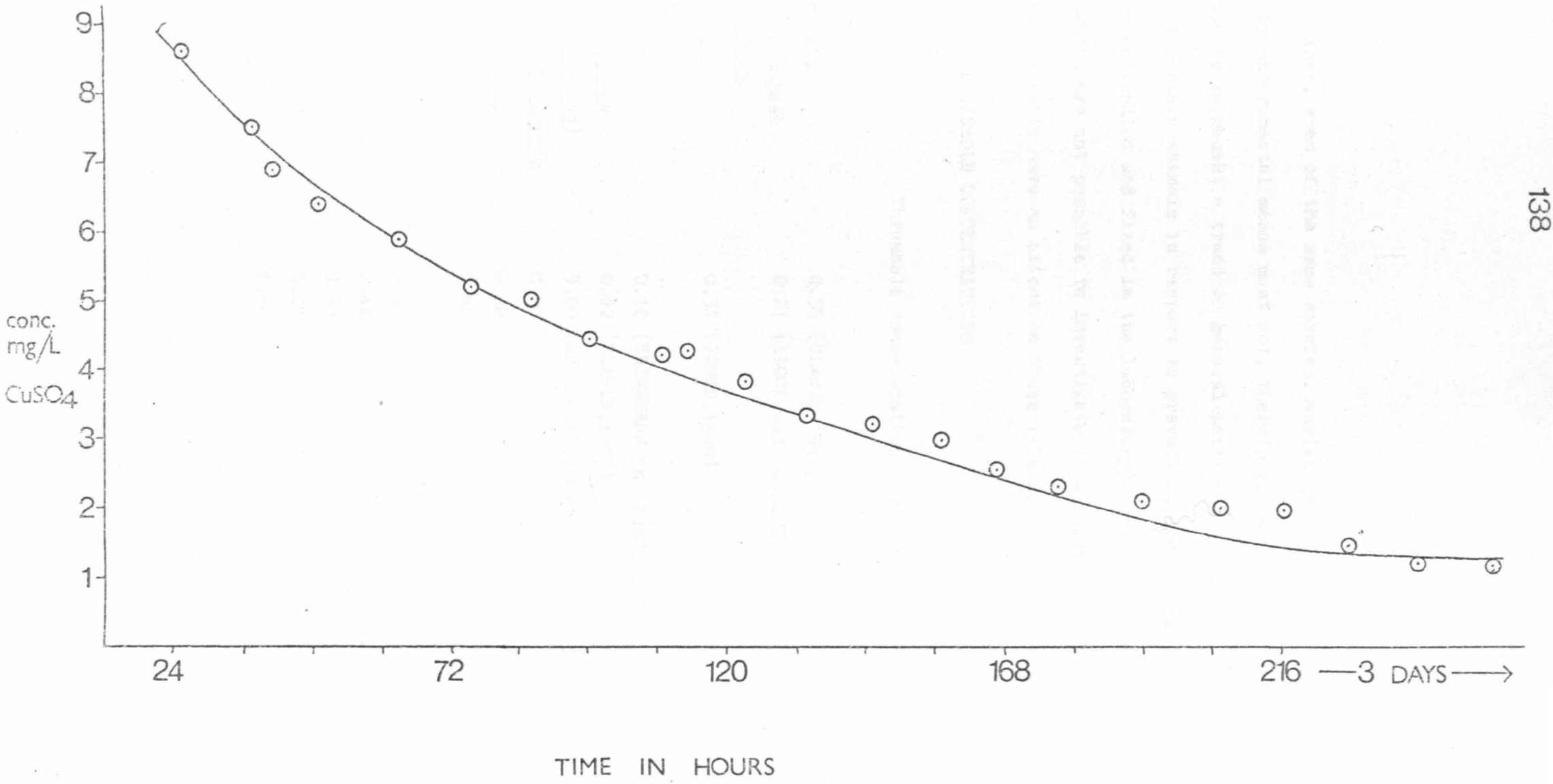


FIG 17 SERIES AA THRESHOLD OF TOXICITY TLM PLOT B.rhodani



that of another, even of the same species, varies greatly. Values obtained by experimental means must not, therefore, be seen necessarily to represent a trend or general pattern representing the response of certain animals in respect to prevailing conditions (which are controlled and fixed in the laboratory), and many other factors, which are not possible to investigate in a study such as this, would probably have an effect on these values.

TABLE 13 THRESHOLD CONCENTRATIONS

Animal	Threshold concentration (mg/l Cu)
MOLLUSCS:	
Mytilus edulis	0.55 (CLARKE,1947)
Mercenaria enigmata	0.21 (KLOCK and PEARSON)
PLATYHELMINTHES:	
Polycelis nigra	0.31 (JONES,1940)
ARTHROPODS:	
Daphnia	0.10 (BRINGMANN and KÜHN,1959)
Balanus balanoides	0.22 (CLARKE,1947)
Crayfish (Orconectes)	3.00 (HUBSCHMAN,1967)
Rithrogena semicolorata	0.76
Ecdyonurus venosus	0.68
Baetis rhodani	0.48
CHORDATES:	
Catfish	0.26
Perch	0.44
Goldfish	0.33
Carp	0.22
Sunfish	0.89

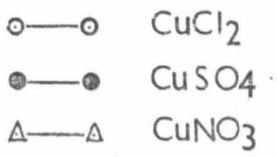
SERIES B

The data obtained from the experiments in this series have been subjected to probit analysis. The method used was as already described and it was used so that it would be possible to compare the effect of three different salts of copper. Any differences in the mode of action will show up more easily if one is dealing with a set of straight lines. Also this type of handling of data requires less experimentation than does the Tlm method. Here only one toxicity test was carried out for each salt at a concentration of 40mg/l copper. Ten animals were used in each test and the actual method was exactly the same as that used in Series A.

Fig.18,A, B, and C shows the results of these toxicity tests and there appears to be little difference in the actions of copper nitrate, copper chloride and copper sulphate. Obviously, the further left the line, the greater is the toxicity since the time value decreases. In the cases of R.semicolorata and B.rhodani copper chloride would appear to be slightly more toxic, but in the graph for E.venosus it appears as the least toxic. These differences are small, however, and do not point to any major effect ascribable to the non-metallic radicles.

It is interesting to note that the lines are not only found in roughly the same position on the graphs, i.e. between 4 and 12 hours, but also that their slopes are similar, showing even more similarity between the toxicities of the three salts since the angle of slope is also an indication of toxicity, i.e. the greater the slope the greater the toxicity.

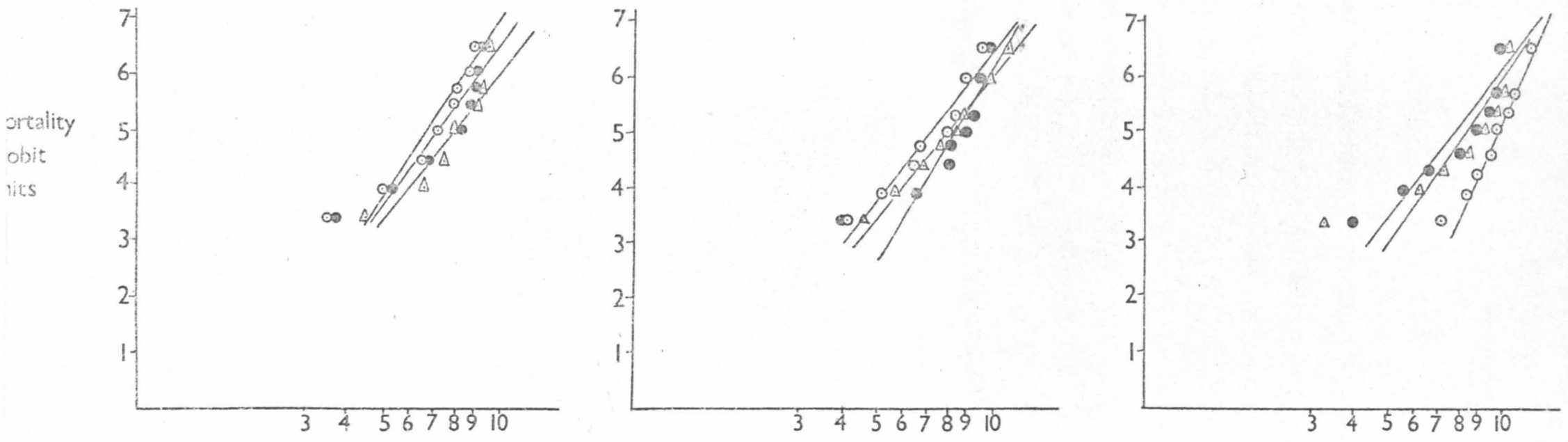
FIG 18 SERIES B PROBIT ANALYSES



A. B. rhodani

B. R. semicolorata

C. E. venosus



It would have been desirable to have carried out more toxicity tests but these are time-consuming and this series did not really have a great deal of importance attached to it. Also, any great difference between the three salts would have been shown up by the experiments which were carried out, thus justifying further investigation into this aspect of toxicity.

The test carried out with magnesium sulphate can also be reported on in this section. This test, using 100mg/l magnesium sulphate with B.rhodani as the test animal, was run for three weeks with no mortality in either test or control animals. The animals in both tanks remained active throughout and there were several moults during the course of the experiment. The experiment was continued after this three-week period but the animals were kept in well aerated beakers. After a period of several weeks there was a 2% mortality in the control animals and a 1% mortality among the test animals.

It is now possible to exclude the sulphate part of the copper sulphate from playing any major role in the toxic action.

The single conclusion that can be drawn from this series is that copper is the main, if not the sole, factor causing a toxic action by some copper containing salts. The non-metallic part seems to have very little effect.

SERIES C

This is a large series and is concerned with the effect of temperature on the basic survival curves for copper sulphate as

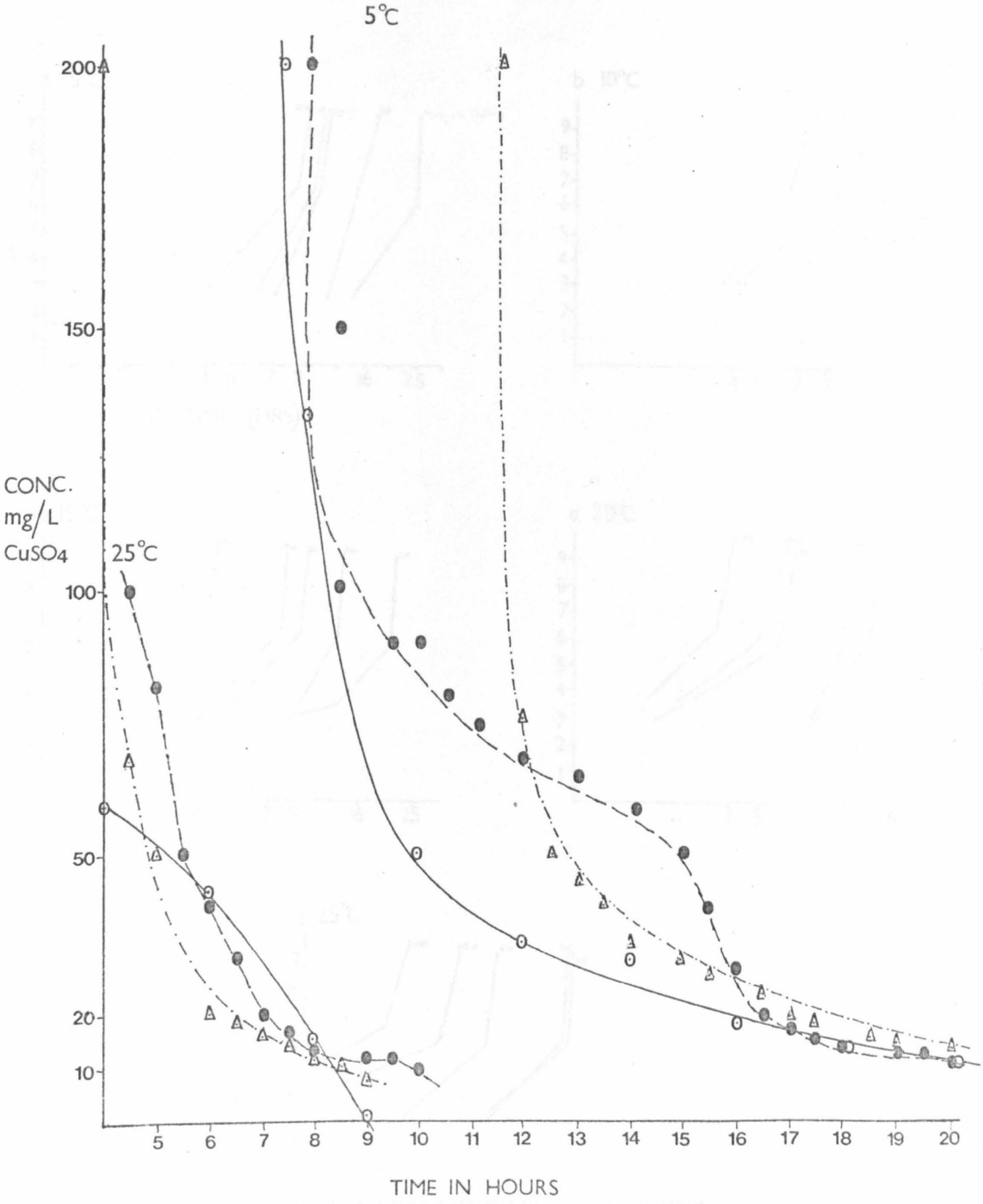
given in Series A. This series is so extensive because toxicity tests have been carried out at different temperatures for each concentration, for each species.

Fig. 19 A and B are the Tlm plots for the three nymph species at different temperatures. From these graphs it is easy to see that the effect of temperature is quite marked, causing a more rapid mortality as it rises.

The data obtained from these tests were also subjected to probit analysis and the results of this procedure from B.rhodani are shown in Fig20 a to e . Here can be seen a gradual shift to the left as the temperature is increased, indicating decreasing survival times. Also, these graphs show that the effect of concentration is accentuated as the temperature begins to rise, this being especially true of concentrations above 20mg/l copper sulphate. In Fig 20a, for example, the lines for 50, 100 and 200 mg/l are fairly close together but as one progresses up the temperature gradient these lines gradually move further and further apart. This is particularly so for the 100 and 200 lines as the behaviour of the lower concentrations is not so clear cut. In the case of B.rhodani these lines are virtually the same at 25°C. A similar pattern emerges when these results are plotted in a slightly different way (Fig21 a to e). Here the lines for temperature are plotted instead of concentration.

It is interesting to note that all the slopes are more or less the same throughout, i.e. they are merely deflected to the left as the temperature or concentration increases, the actual angle is not so uniformly affected. If one equates angle of slope with degrees

FIG 19 SERIES C EFFECTS OF HIGHEST AND LOWEST EXPERIMENTAL TEMPERATURES ON SURVIVAL CURVES



B.rhodani; E.venosus; R.semicolorata

FIG 20 SERIES C PROBIT ANALYSES FOR B.rhodani (temperature)

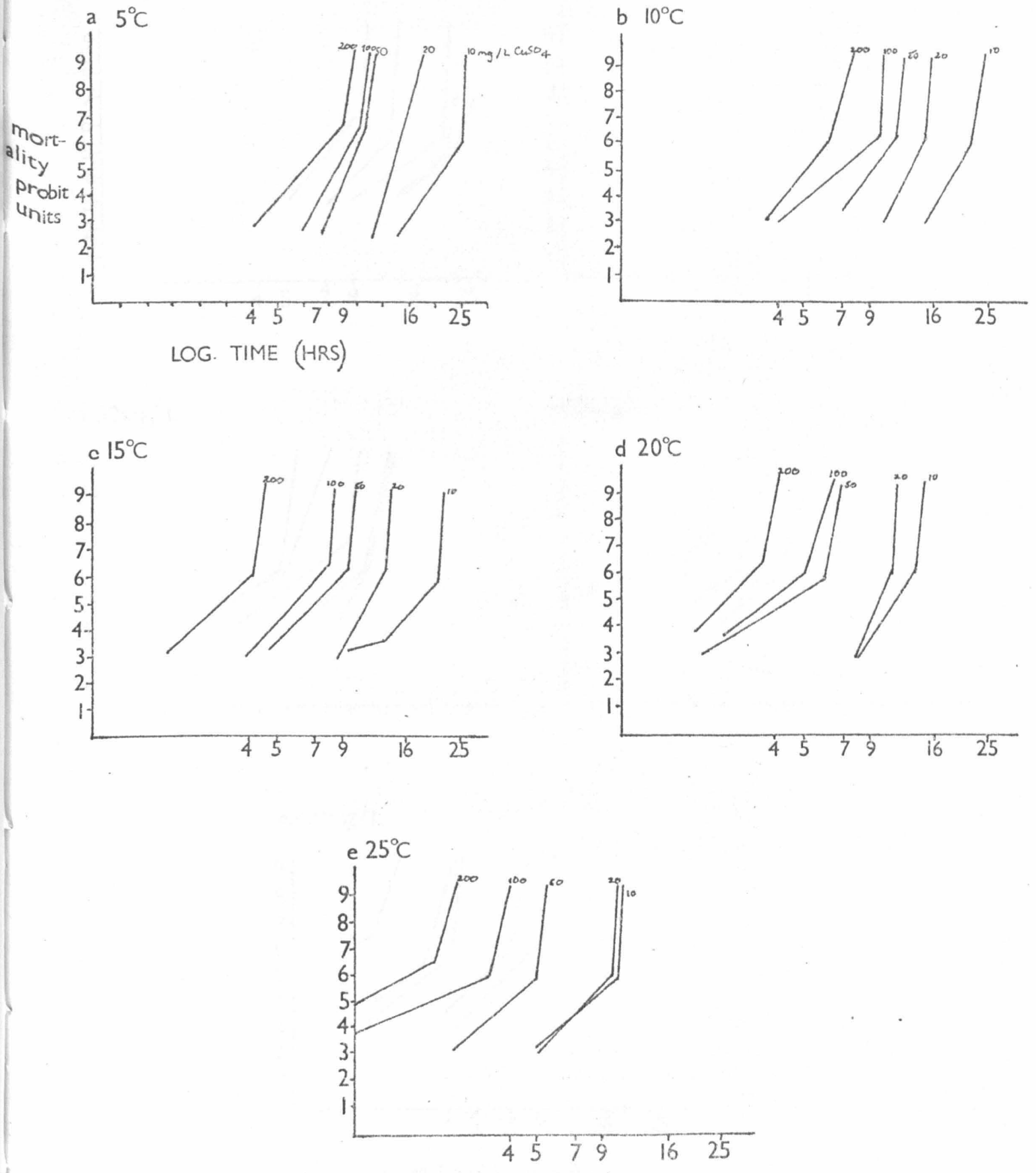
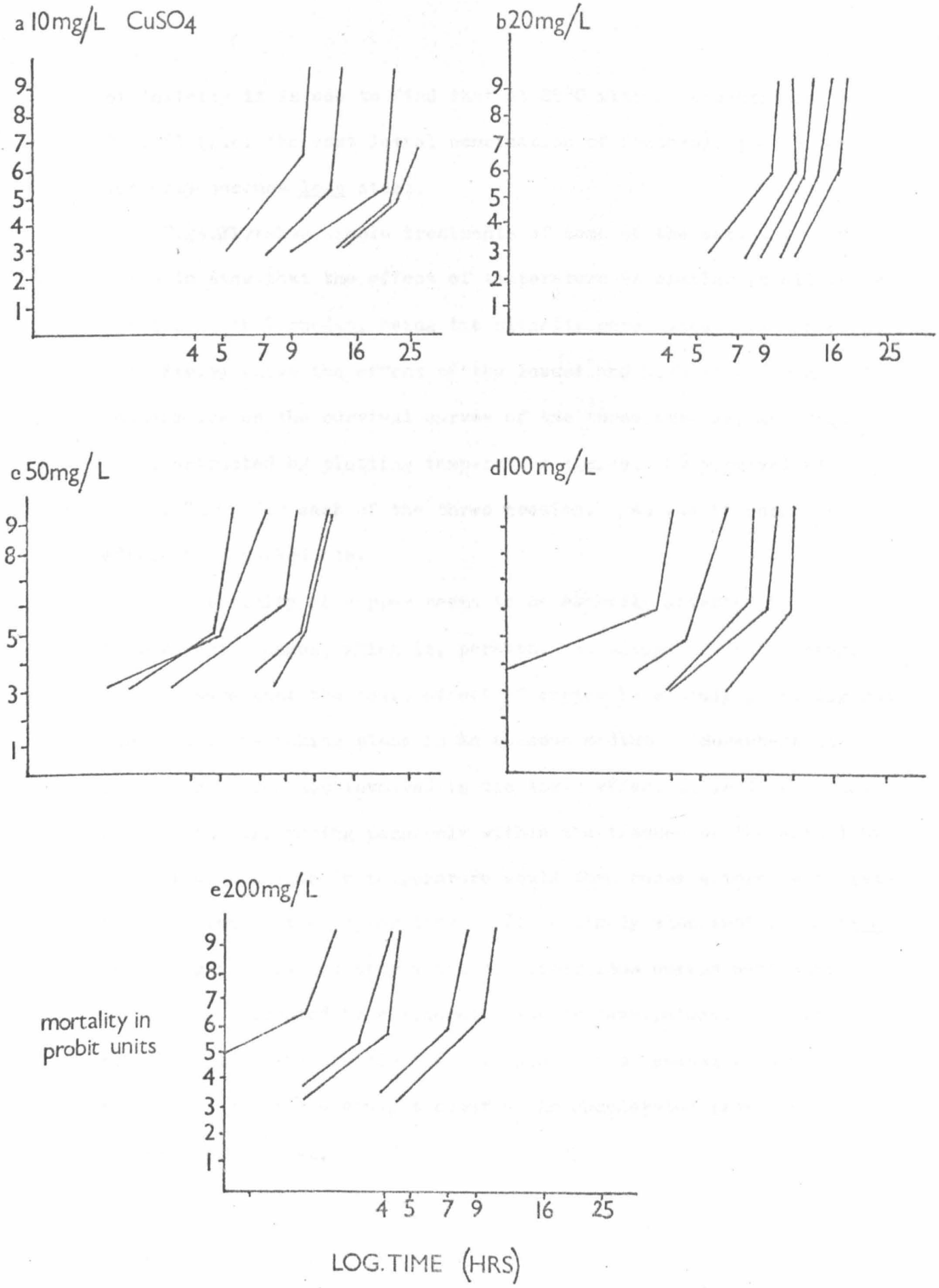


FIG21 SERIES C PROBIT ANALYSES FOR B.rhodani (concentration)



of toxicity it is odd to find that at 25°C with a concentration of 200mg/l (i.e. the most lethal combination of factors), the slope actually becomes less steep.

Figs.20,21 show simple treatments of some of the data but they do serve to show that the effect of temperature is similar in all three species, with B.rhodani being the slightly more susceptible species.

Fig.19 shows the effect of the lowest and highest experimental temperature on the survival curves of the three species, and Fig. was constructed by plotting temperature against 50% survival at 50mg/l $CuSO_4$ for each of the three species. As can be seen, the effect is a marked one.

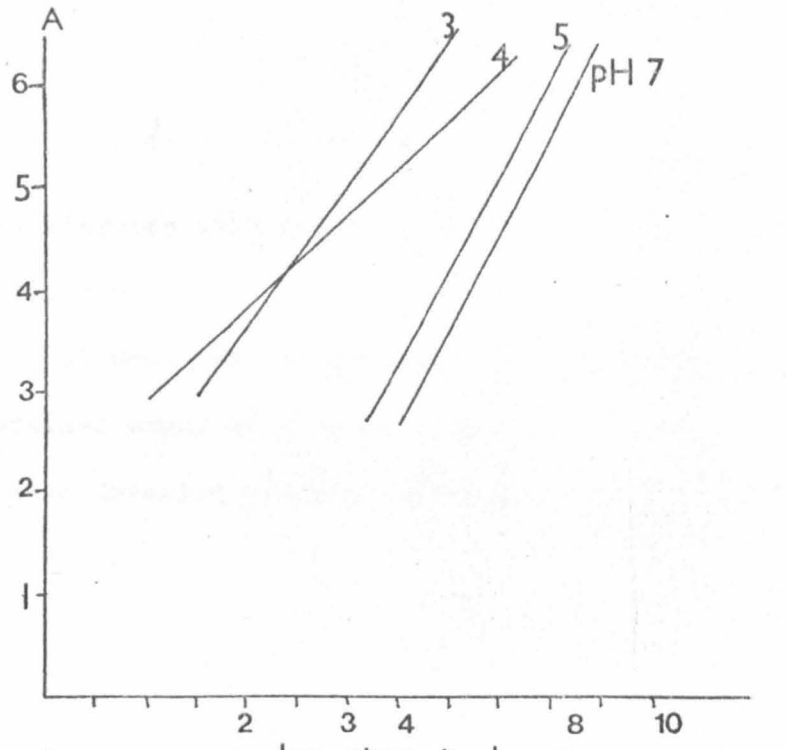
The toxicity of copper seems to be markedly affected by temperature changes, which is, perhaps, not altogether surprising. If we assume that the toxic effect of copper is a truly physiological one then it is taking place in an aqueous medium. Somewhere in the process or processes involved in the toxic effect it is likely that copper ions are moving passively within the tissues of the animal by diffusion. A rise in temperature would then cause a more rapid rate of diffusion of the copper ions. It is likely also that any active mechanism for the transportation of copper ions across membranes would be accelerated by a moderate rise in temperature. It is possible that irreversible and detrimental biochemical changes involving copper are brought about at an accelerated rate due to higher temperatures.

SERIES D

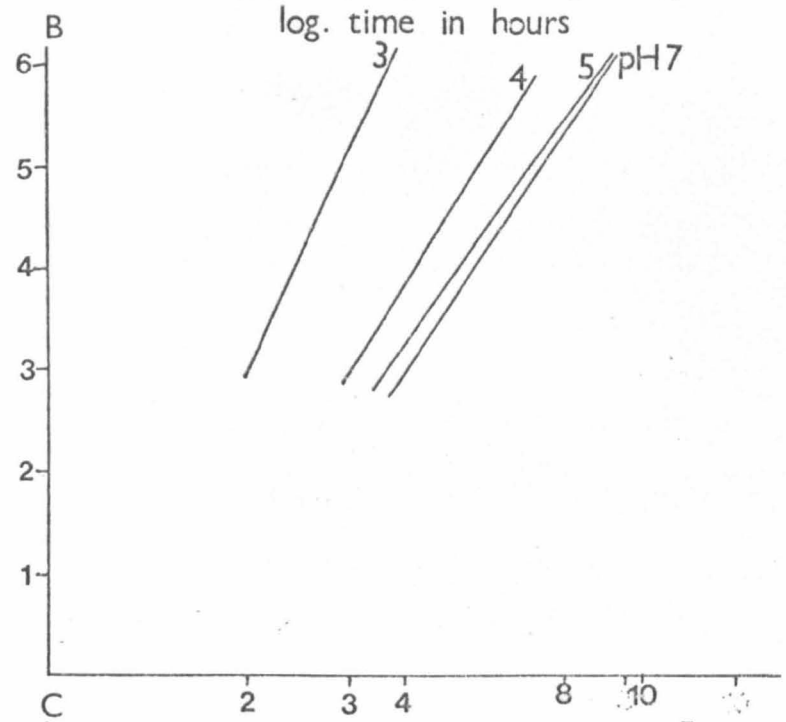
Graphs 22.A to C show that pH does have an effect on the toxicity of copper to the three species of Mayfly used. Between pH5 and pH7 the effect is very slight but below pH5 the toxic effect of copper is accelerated appreciably. Without knowing more about the details of the actual mechanism of copper toxicity it is difficult to offer any explanations as to why exactly lower pH values have this effect. At pH values as low as 3, however, it is possible that this in itself will have an adverse effect on the animal and in some way lower its tolerance to the toxicant. pH is an important factor in most physiological mechanisms since the enzymes which control them depend on fairly specific pH values for their correct functioning. For animals in an aquatic medium drastic changes in the pH of their environment could surpass the limits within which compensating inbuilt buffering systems are able to function effectively. This could explain why a value of pH5 does not appear to have a very significant effect on the nymph's response to copper. Also, while the hydrogenion concentration of extra-cellular fluids is generally near or above pH7, the interior of most cells is below pH7 and the average isoelectric point of cell proteins corresponds to a value of pH5.

At low pH values, therefore, it is possible that internal buffering systems break down so that the correct pH values for the functioning of major enzyme-controlled processes are no longer maintained. One of these processes could be concerned with the active removal and excretion of excess ions, such as copper.

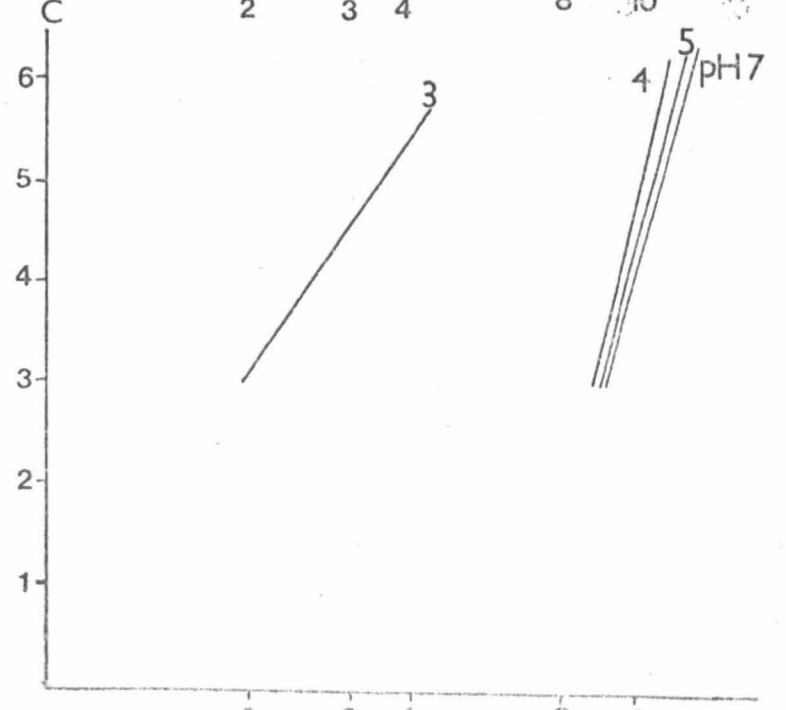
FIG 22 SERIES D PROBIT ANALYSES



B.rhodani



R.semicolorata



E.venosus

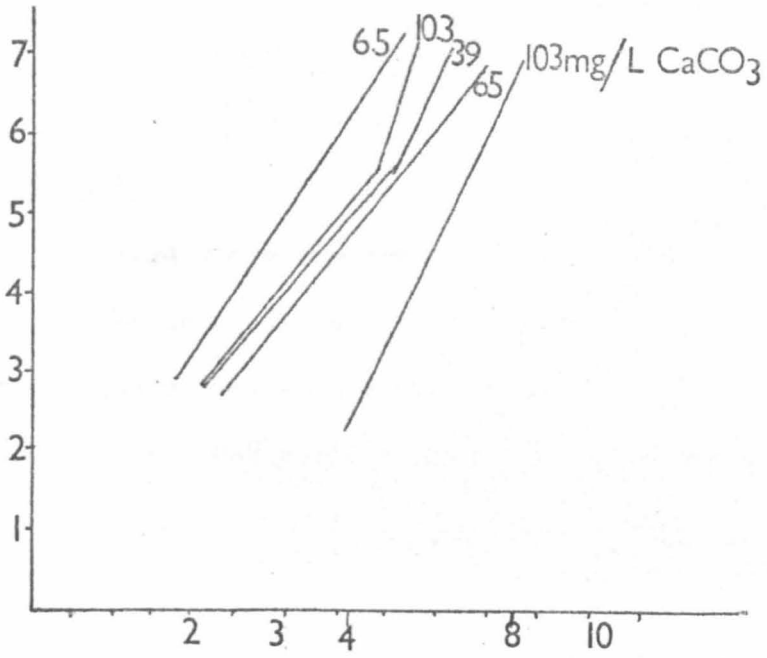
Interference with such a process would then produce a much reduced tolerance.

It would be interesting to extend this section to a more detailed study of a range of pH values between pH5 and pH7 to give a more detailed picture of the effects of small changes in pH on copper toxicity.

SERIES E

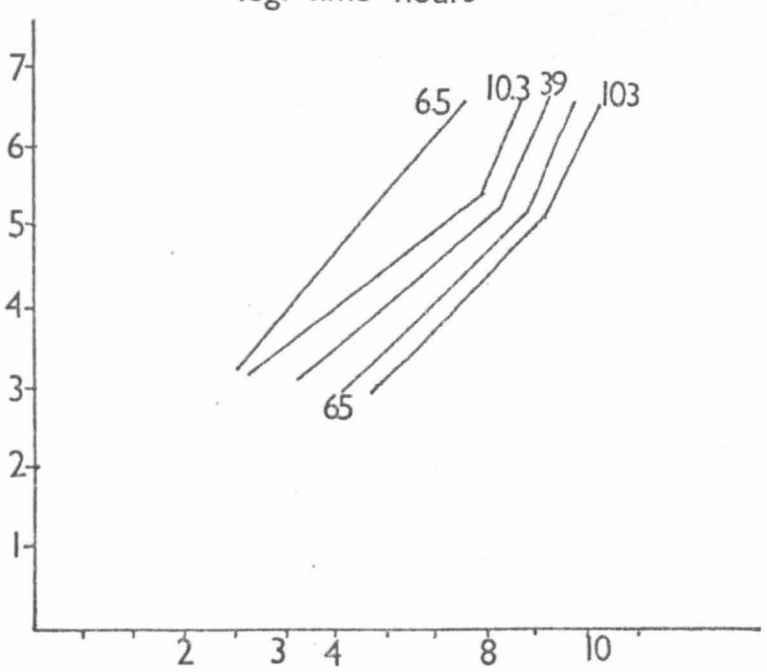
As can be seen, the graphs (Fig23) show that increased hardness tends to lessen the toxic effect of copper. That heavy metals are less toxic in hard waters has been known for some time now (POWERS,1939), and the effect is probably due to an antagonistic effect between the ions causing the hardness, and the heavy metal ions. In the case of these experiments the respective hardnesses were obtained by increasing the amounts of calcium and magnesium salts in the test waters. It has been shown already (JONES,1938; HERBERT and WAKEFORD,1962) that high calcium concentrations reduce the toxicity of heavy metals to organisms and the results obtained here would seem to show that this relationship holds true in the case of calcium (and possibly magnesium) and copper.

FIG 23 SERIES E PROBIT ANALYSES



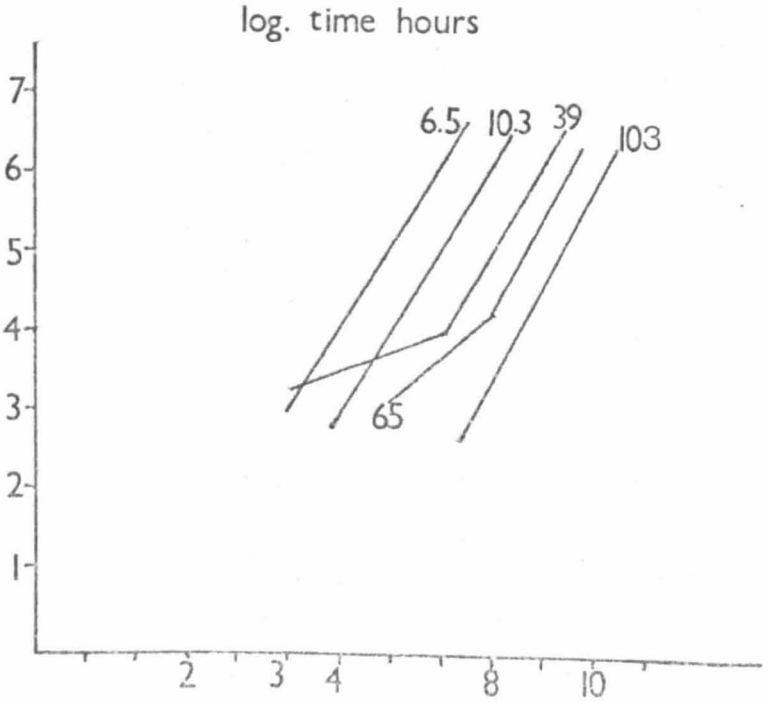
mortality probit units

B.rhodani



mortality probit units

R.semicolorata



mortality probit units

E.venosus

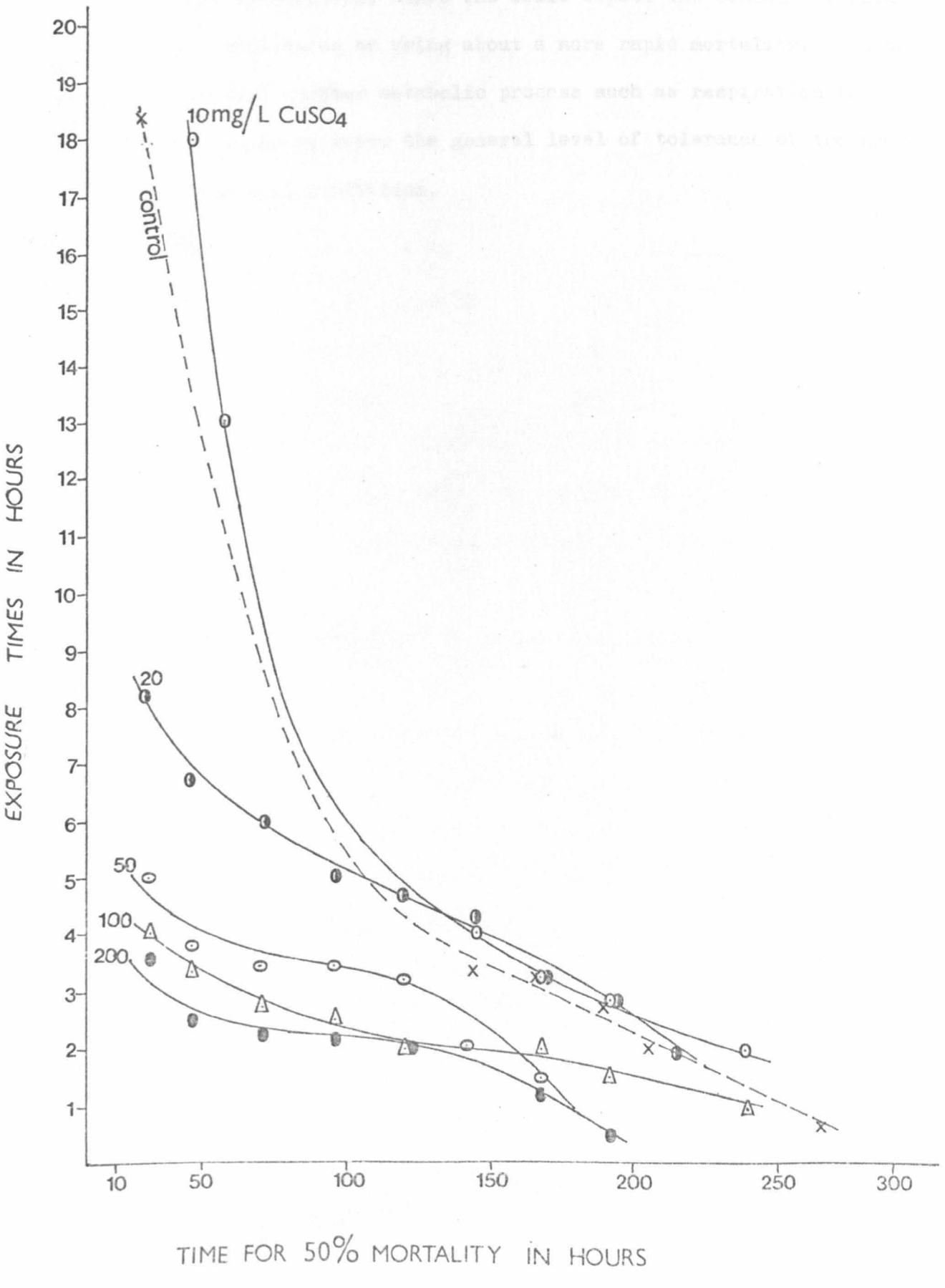
log. time hours

SERIES F

Fig.24 shows the graph obtained from the data for this series. As can be seen, there is a surprising difference in effect between the exposures made at 10mg/l and 20mg/l copper sulphate. Also, some of the test animals made a recovery after removal from the copper solution, removal merely prolonging the time taken for the animals to be killed. As can be seen from the curves obtained, the greater the concentration the shorter this period is, and it is interesting that the concentrations used should cause this ordered effect even after the animals had been removed from the toxic solutions.

The fact that even short exposures to copper at low concentrations causes the death of the nymphs over a protracted period is interesting because it seems to suggest that copper is not being excreted after removal from the toxic solutions. It is therefore possible that copper is causing a blocking, or at least partial blocking, of urine formation which is not as extreme as in the case of nymphs subjected to prolonged exposure but severe enough to bring about death possibly as a result of a gradual building up of harmful waste products. In the case of exposure to the lowest concentration for a short period of time the question of copper-induced mortality is not so clear cut. Here 100% mortality of test animals is brought about after a period of about 37 days but by then control animals are also beginning to die (see Fig.24). It is possible, therefore, that at concentrations lower than 10mg/l copper sulphate nymphs will recover provided the exposure is not a long one.

FIG 24 SERIES F TLM CURVES B.rhodani



Whether at low concentrations urine formation is being affected is more open to question, since one would expect the consequent build-up of toxic substances to bring about a more rapid mortality. It is possible that another metabolic process such as respiration is affected so as to lower the general level of tolerance of the nymphs to experimental conditions.

SUMMARY

The findings of this section can be summarized as follows:

1) Tlm survival curves have been determined for the species E.venosus, R.semicolorata and B.rhodani. Of these, B.rhodani appears to be the most susceptible species.

2) The thresholds of toxicity of copper have been found for each species. These are as follows (as mg/l copper):

R.semicolorata	0.76
E.venosus	0.68
B.rhodani	0.48

These figures are relatively high when compared to values available for other organisms and show these nymphs to be comparatively tolerant to abnormally high concentrations of copper.

3) Copper has been shown to be largely responsible for the toxic effect of copper salts.

4) There is a direct relationship between an increase in temperature and an increase in the toxic action of copper.

5) There appears to be little difference in the toxicity of copper in solutions from pH7 to pH5. pH values lower than 5, however, do result in a more pronounced toxic action.

6) The toxicity of copper is reduced in harder waters.

7) Even short exposures to copper concentrations of 20mg/l copper sulphate and above bring about the death of test animals after they are removed from the copper solutions. The mortality rate here is drawn out over a much protracted period of time, however.

Chapter 6

HISTOCHEMISTRY

This section of the investigation is concerned with establishing that copper is actually entering the body of the nymphs exposed to copper containing solutions. The cuticle of Mayfly nymphs is impermeable and the most likely method of entry of copper is by way of the mouth, either by active drinking or when the nymphs feed. If this is so, then it should be possible to detect copper at least within the lumen of the gut. This in itself would not, however, be evidence that copper was being taken up by the nymphs unless some evidence of absorption of copper through the gut wall could be obtained. The most direct method of obtaining such evidence seemed to be to use histochemical techniques where a stain that was specific to copper could be employed to provide visual evidence of the presence of copper in any of the organs of the nymphs. This, it was hoped, would give a rough picture of the main areas, at least, of accumulation of high concentrations of copper, and that this could be the basis of a more detailed physiological study of the problem.

Materials and methods

Nymphs which had been used as test animals in the previous series of toxicity tests were used for this investigation. These were fixed in a formaldehyde-saline fixative which was made up as follows:

Formalin (full strength)	$10cm^3$
Sodium chloride, 10% aqueous	$7cm^3$
Distilled water	$83cm^3$

This fixative has been found useful for general histochemistry.

The fixed nymphs were then paraffin embedded prior to section cutting. In order to do this it was necessary to dehydrate the tissue, and the following procedure was adopted: after washing the fixed nymphs in distilled water they were left for one hour in each of the following: 50% ethanol, 90% ethanol, absolute ethanol (2 lots, one hour each, absolute ethanol-xylene (1:1)), xylene.

After this treatment the nymphs were placed in melted paraffin and kept in an oven at a temperature of 60°C. The molten paraffin was kept in solid watch glasses which had been previously smeared with 5% glycerol in 60% ethanol to prevent sticking. One nymph was placed in the centre of the watch glass in the paraffin by means of heated forceps. By blowing gently on the surface of the paraffin a thin crust was made to form and then the whole watch glass was slid below the surface of cold water in a dish. By doing this the paraffin block floated out of the watch glass after a few minutes. When solidified it was removed from the water and left for not less than 24 hours before sectioning.

This procedure was used for all the nymphs used for sectioning.

Serial sections along the longitudinal axis of the body were cut, using a rocking microtome, beginning at the anterior end of the nymphs. In addition to this longitudinal sections were also made. The visual evidence shown in the plates was taken from a single nymph but the results obtained were typical of those obtained from several other specimens of E.venosus.

Staining

There are several histochemical stains which react with copper, and because of the ease with which it can be used Rubeanic acid (dithio-oxamide (CS.NH)) was selected. This detects copper and copper complexes by staining them black. Since the sections were paraffin-embedded the following procedure had to be adopted. The sections were dewaxed in xylene and then placed face down over a beaker of concentrated acid for fifteen minutes. The following solutions were necessary:

1. Rubeanic acid 50mg

   Absolute alcohol $50cm^3$ (this was the rubeanic acid stock solution)

2. Sodium acetate solution

   Sodium acetate 10g

   Distilled water $100cm^3$

From these two solutions a staining solution was made up as follows:

Rubeanic stock solution $2.5cm^3$

Sodium acetate solution $50cm^3$

The staining procedure can be summarized as follows:

1. All sections were brought down to water.
2. The sections were then placed in the staining solution at 37^oC overnight.
3. The solutions were then transferred to 70% alcohol for fifteen minutes.
4. The sections were placed in absolute alcohol for six hours.
5. Finally, the sections were placed in xylene and mounted in Canada Balsam.

This method is based on HOWELL,1959, after OKAMOTO and UTAMURA.

Results

Plates 1 to 29 show the evidence obtained by using histochemical techniques. The distribution of copper in the nymphs of E.venosus can be grouped into these areas:

1. The alimentary canal
2. The nervous system
3. Other organs

Fig.25 is a diagrammatic representation of these areas showing the approximate levels of the sections from which the photomicrographs shown in the plates were taken. Each section is given an index number which has the prefix T (transverse) or L (longitudinal). These indices are referred to in the descriptions that follow and it is hoped that they are of some help in placing the sections within the general body plan of the nymph. Each plate number is, therefore, followed by the index number for that section.

1. The alimentary canal

The main region of copper accumulation in the alimentary canal is in the region of the mid gut. No copper was found in the fore gut (oesophagus) which is here, a structure which serves merely to conduct food from the mouth to the digestive and absorbing regions of the mid gut. The fact that no copper was found here may be due to two factors.

a) The oesophagus in insects is lined with cuticle and is therefore impermeable. This means that accumulation cannot be taking place in the cells of the wall of the oesophagus. The only place

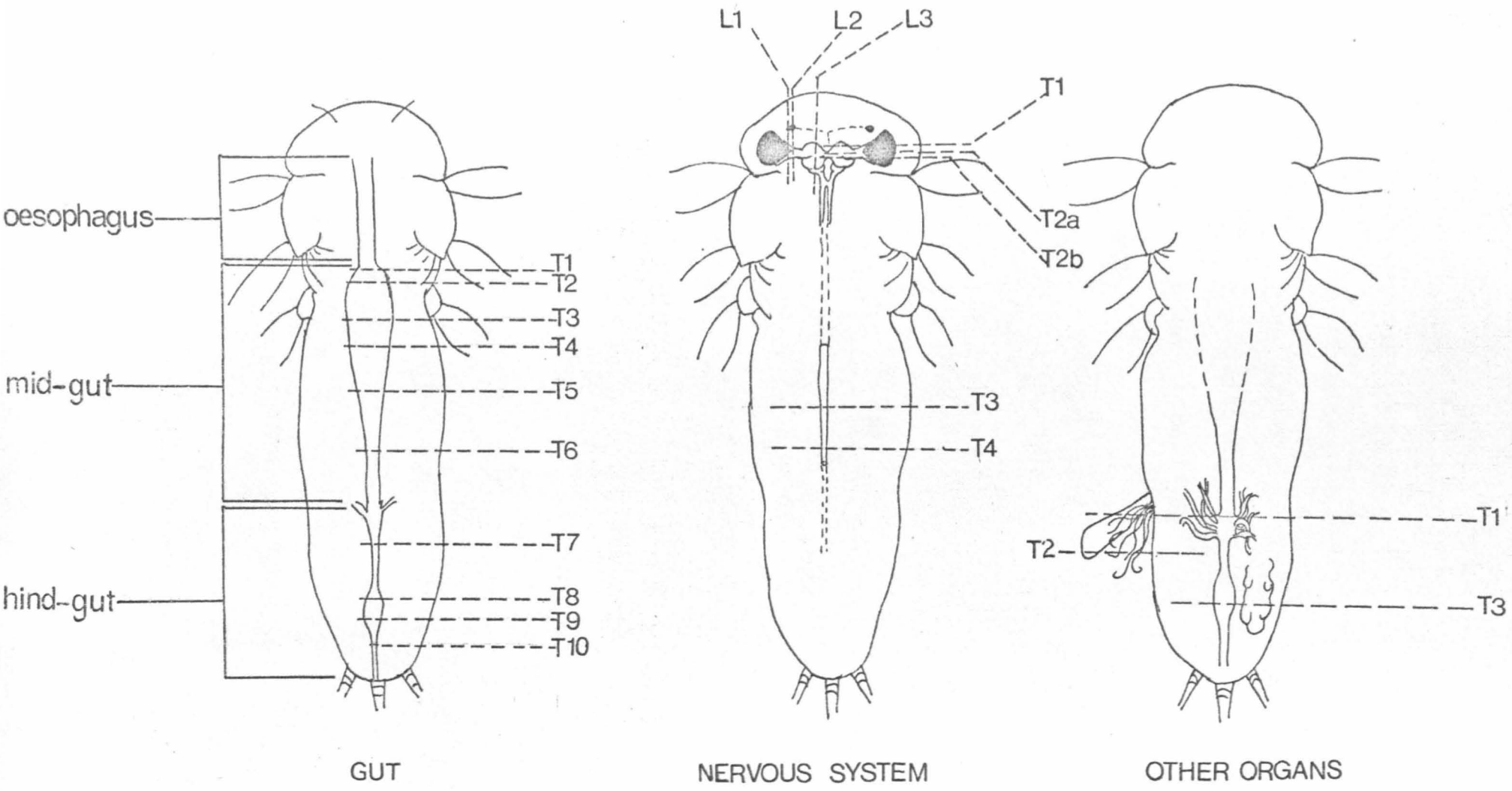


FIG 25 COPPER DISTRIBUTION IN E.venosus (rubeanic acid)

where copper could be expected to be found in this region is, therefore, within the lumen. Ingested material probably remains in the oesophagus for a relatively short period of time so that unless the nymphs are feeding or drinking continuously there is not much likelihood of finding very much material in the oesophagus, unless the nymphs were killed immediately following ingestion of food or water.

b) Any copper which is within the lumen of the oesophagus is not at a high enough concentration to be detected by the rubeanic acid. The small amounts of copper in this region may be a result of the reasons given in (a) above.

On reaching the level of the mid gut the presence of copper is detectable. Plate 1 (T1) shows large accumulations of copper in both the lumen and the cells of the gut wall. This is the digestive region of the alimentary canal and material tends to remain here for longer periods of time. A similar picture is obtained a little further along the mid gut region - Plate 2 (T2), and Plate 3 (T3).

Further back, it is interesting to note that the lumen itself begins to clear of copper and the majority of the detectable copper is in the cells of the gut - Plate 4 (T4). This is seen particularly clearly in Plate 5 (T5). The explanation of this is that this part of the gut is probably mainly responsible for the absorption of the products of digestion and copper is also being actively absorbed and so is removed from the lumen of the gut and becomes concentrated in the cells of the gut wall - Plates 6 (T6) and 7 (T6).

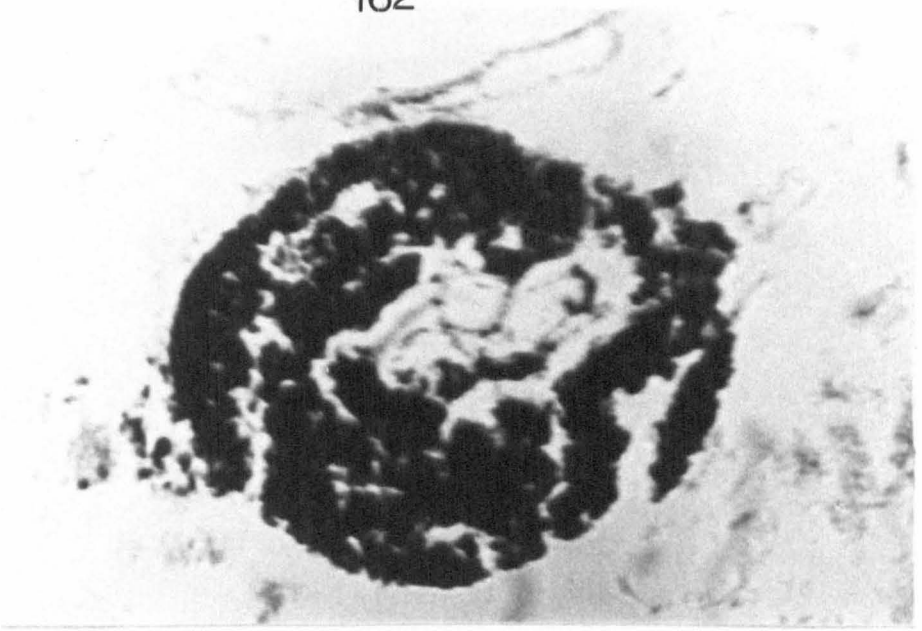


PLATE 1 (T1) MID-GUT E.venosus x100

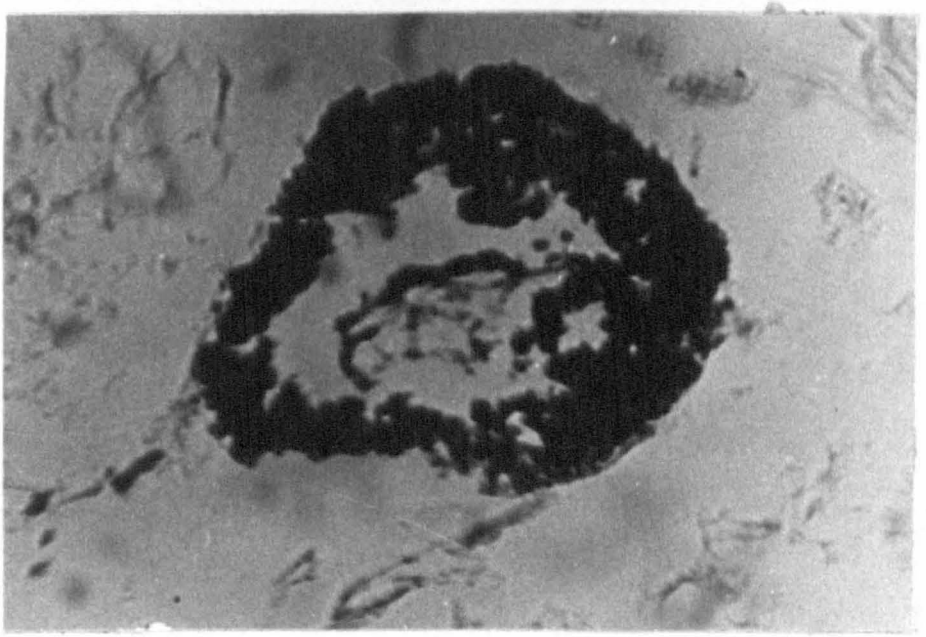


PLATE 2 (T2) MID-GUT E.venosus x100

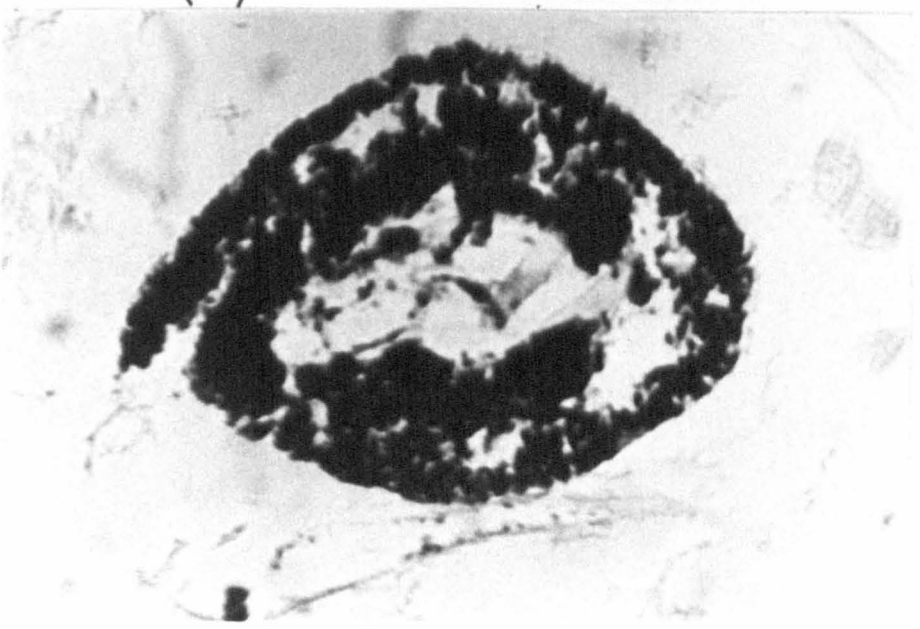


PLATE 3 (T3) MID-GUT E.venosus x100

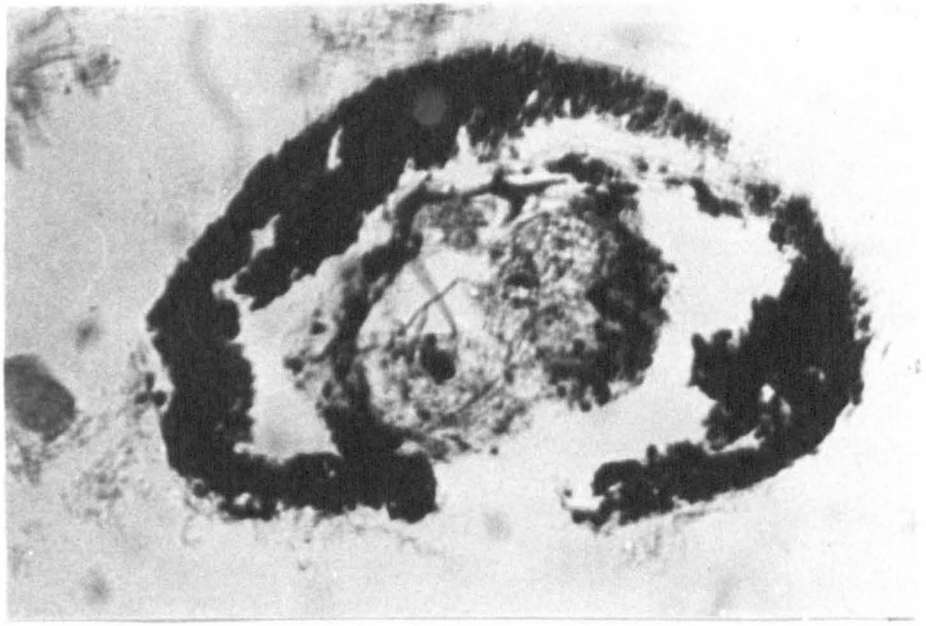


PLATE 4 (T4) MID-GUT E.venosus x100

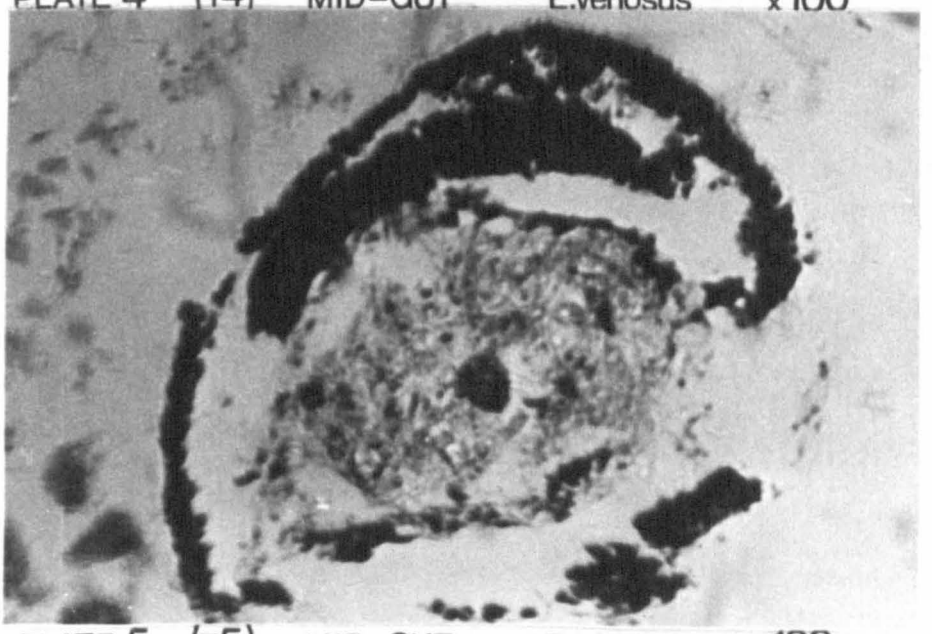


PLATE 5 (T5) MID-GUT E.venosus x100

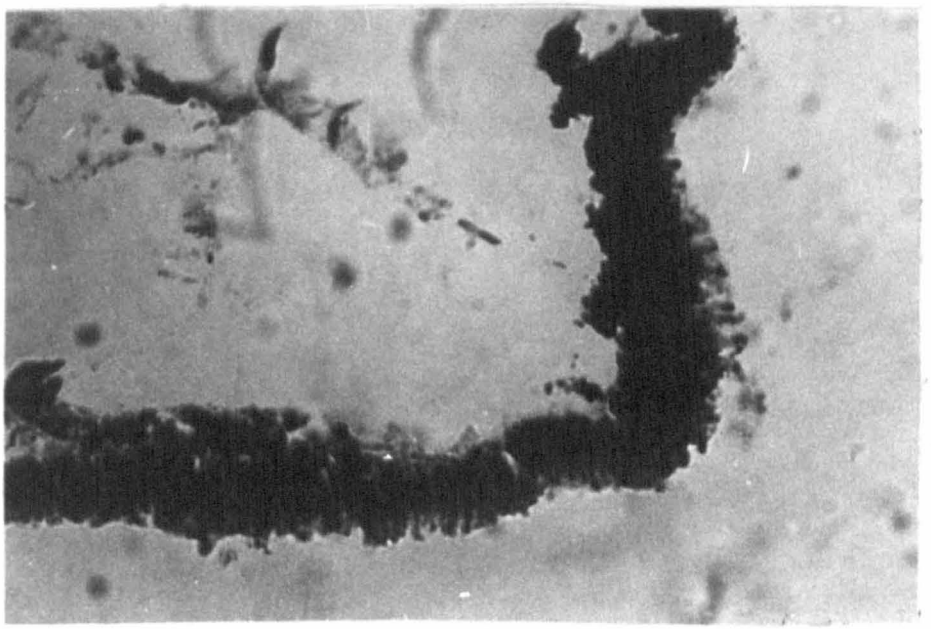


PLATE 6 (T6) GUT-WALL E.venosus x200

The absorption of copper is of great interest since it suggests that for a time, at least, copper is being taken up physiologically by the nymphs and is not merely being ingested into the lumen of the gut (which, strictly speaking, must be considered as being outside the animal) and then egested. Absorption also suggests the possibility of assimilation with all the implications that this carries in terms of possible true physiological effects of copper.

In the region of the hind gut the level of copper detected falls off drastically - Plate 8 (T7), Plate 9 (T8) and Plate 10 (T9). In the terminal portion of the hind gut there seems to be very little copper - Plate 11 (T10).

This apparent disappearance of copper in the hind gut can be explained in terms similar to those described in explanation for the failure of rubeanic acid to detect copper in the oesophagus. This is because the hind gut is similar to the oesophagus in that it, too, has a cuticular lining and is, therefore, also impermeable. One would not, therefore, expect to find copper in the cells of the wall of the hind gut. Small amounts of copper are detectable in the lumen, however, which suggests that small amounts of copper are going through the alimentary and are presumably being egested with other material being removed from the body. The presence of copper in small amounts in this region may be further explained by the possibility of active excretion of this ion taking place. The excretory organs, the malpighian tubules, concentrate waste metabolic products from the haemocoel and expel these directly into the hind gut. These waste products are not stored here for any great period

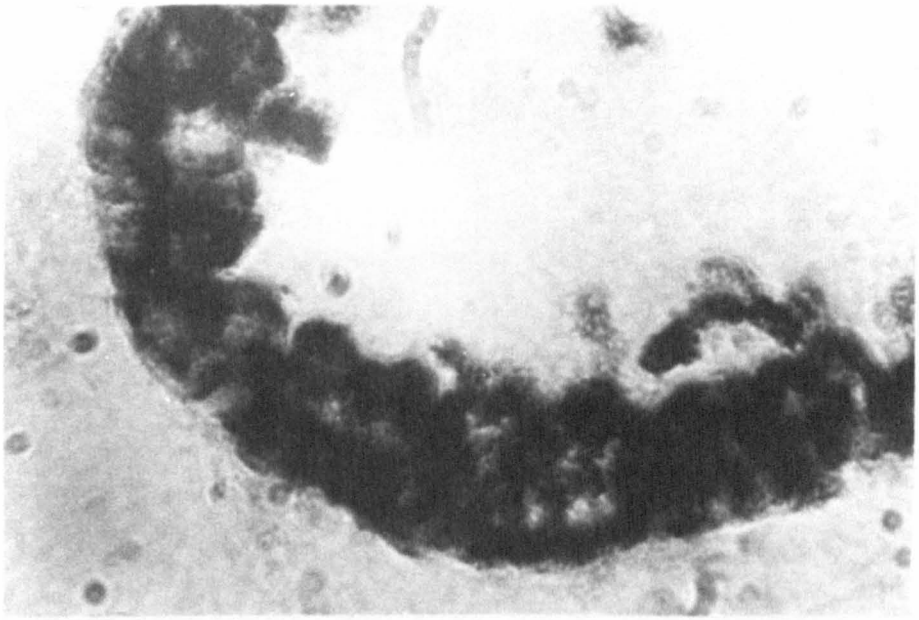


PLATE 7 (T6) GUT WALL E.venosus X400

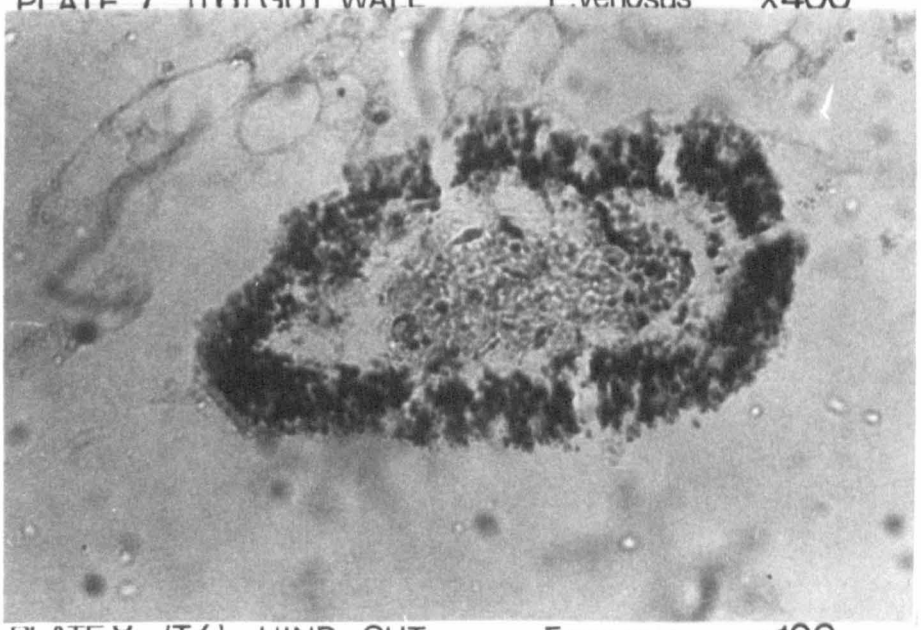


PLATE 8 (T7) HIND-GUT E.venosus X 100

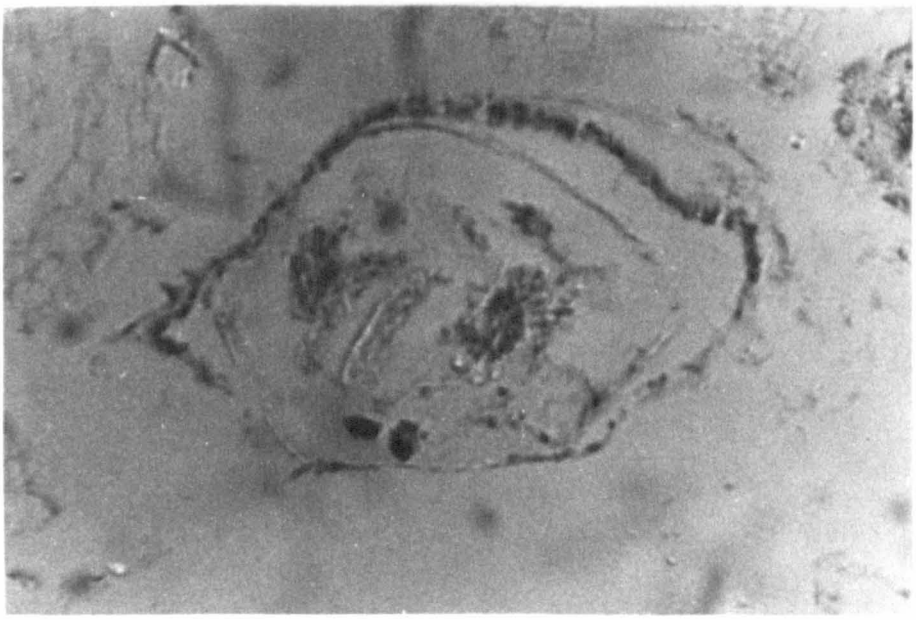


PLATE 9 (T8) HIND-GUT E.venosus X 100

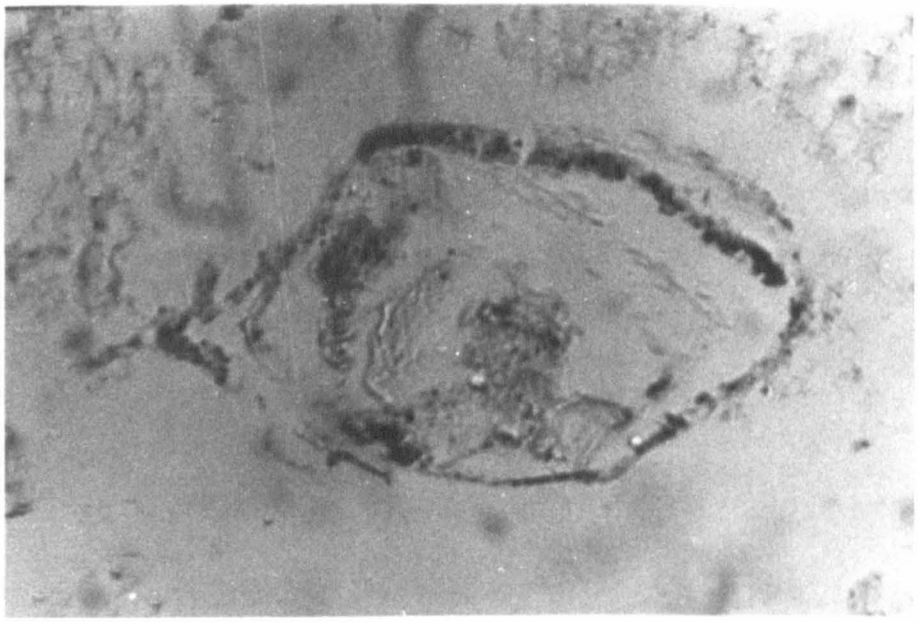


PLATE 10 (T9) HIND-GUT E.venosus X100

PLATE 11 (T10) HIND-GUT E.venosus X 100

of time so that accumulation giving a stronger reaction with the rubeanic acid would not have time to occur.

The distribution of copper in the alimentary canal of E.venosus can be summarized in the following table. Here the presence of copper as detected by rubeanic acid is indicated by +, on a scale of 0 to 5, giving some indication, if a somewhat crude one, of the relative amounts involved and their spacial distribution.

TABLE 14 DISTRIBUTION OF COPPER IN THE ALIMENTARY CANAL OF E.VENOSUS

Region of gut	Index	Lumen	Cells of wall	Plate
Oesophagus	-	-	-	-
Mid-gut	T1	+++++	+++++	1
	T2	+++	+++++	2
	T3	+++	+++++	3
	T4	++	+++++	4
	T5	+	+++++	5
	T6	-	+++++	6
		-	+++++	7
Hind-gut	T7	+	+++	8
	T8	+	++	9
	T9	+	+	10
	T10	+	+	11

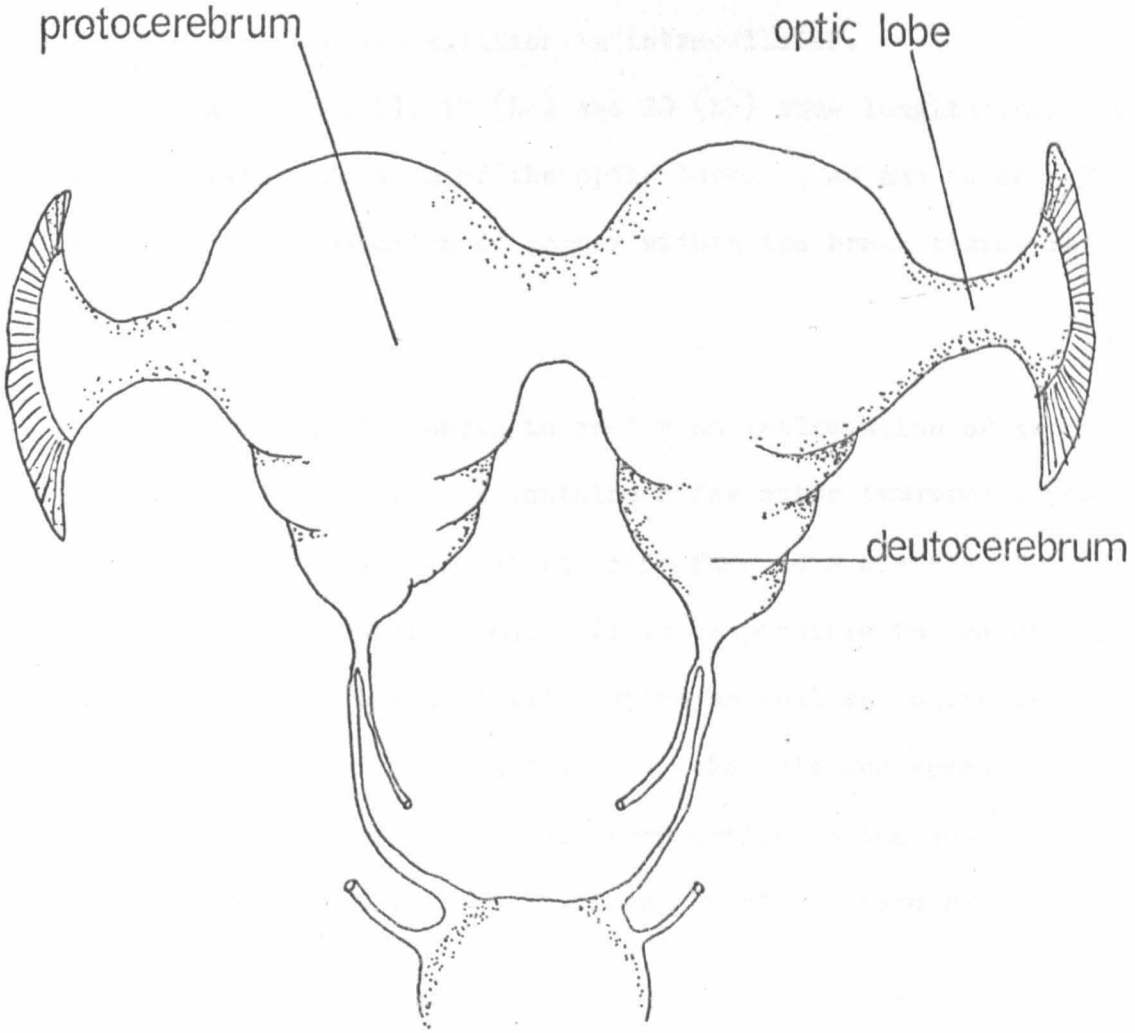
The results obtained using rubeanic acid therefore show that copper is:

1. being taken into the gut of the nymphs of E.venosus.
2. being absorbed by the cells of the wall of the mid-gut.
3. possibly being actively excreted.

The amounts involved appear to be large although it is not possible, using this technique, to give any quantitative statement of absolute amounts. This evidence does, however, strongly suggest an internal action of copper toxicity, especially since no evidence was found of any surface adsorption of copper on any external surface of the nymphs.

2. The nervous system

Fig 26 is a diagram of the brain of a typical insect and is similar to that of E.venosus.



The distribution of copper in the brain is seen to be confined to bands in the optic lobes and in more diffuse areas of the protocerebrum and deutocerebrum. Plate 12 (T1) shows a relatively thick band of copper within the tissues of the optic lobe and extending towards the median region of the brain. Plate 13 (T2a) shows that this band persists into the deeper tissue of the optic lobe, and Plate 14 (T2b) that the copper continues into the protocerebrum region of the brain. (Incidentally, Plate 14 also shows a section of the oesophagus which shows the absence of copper in this region of the alimentary canal.) Plate 15 (T3) shows that copper is present in a similar band in the other optic lobe, and Plates 16 (T3) and 17 (T3) are higher magnifications of this band in the left optic lobe, showing that the accumulation is intracellular.

Plates 18 (L1), 19 (L2) and 20 (L3) show longitudinal sections of the brain and parts of the optic lobes. As can be seen from these, the accumulation of copper within the brain tissue is extensive.

The brain of insects is really an amalgamation of supra-oesophageal ganglia. It contains a few motor neurones concerned with antennal movements but its main functions are sensory and those concerned with coordination. It is responsible for maintaining the general tonus of the skeletal muscles as well as controlling the local reflexes which are mediated by thoracic and abdominal ganglia. Also, the brain exerts an inhibitory action on the sub-oesophageal ganglion which has the effect of suppressing unnecessary movements.

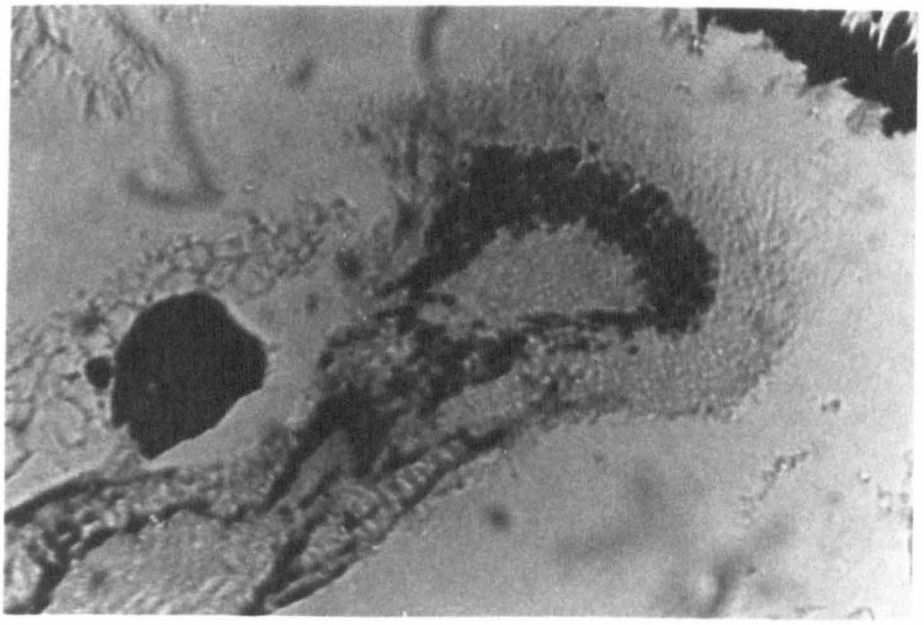


PLATE 12 (T1) OPTIC LOBE x 100

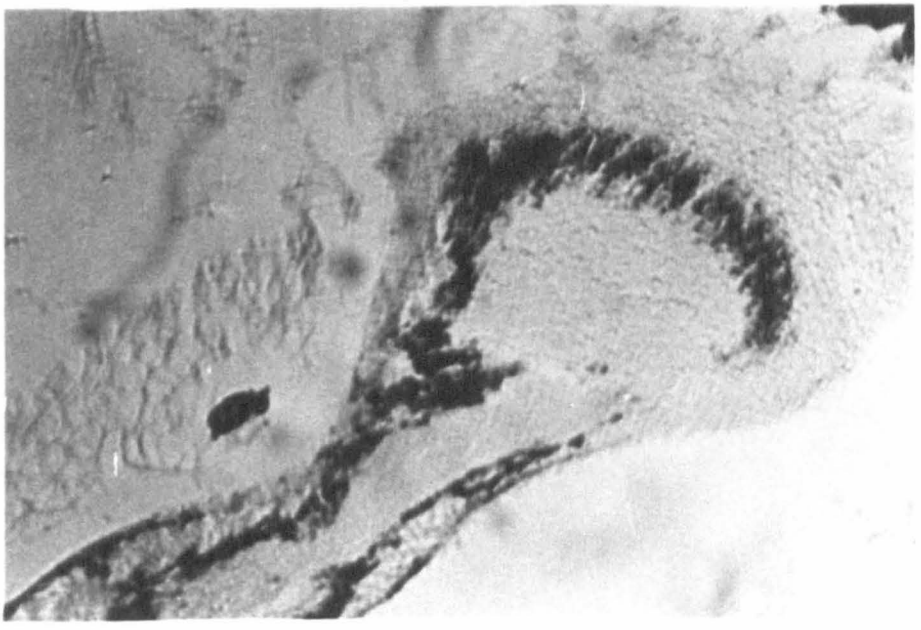


PLATE 13 (T2a) OPTIC LOBE x 100

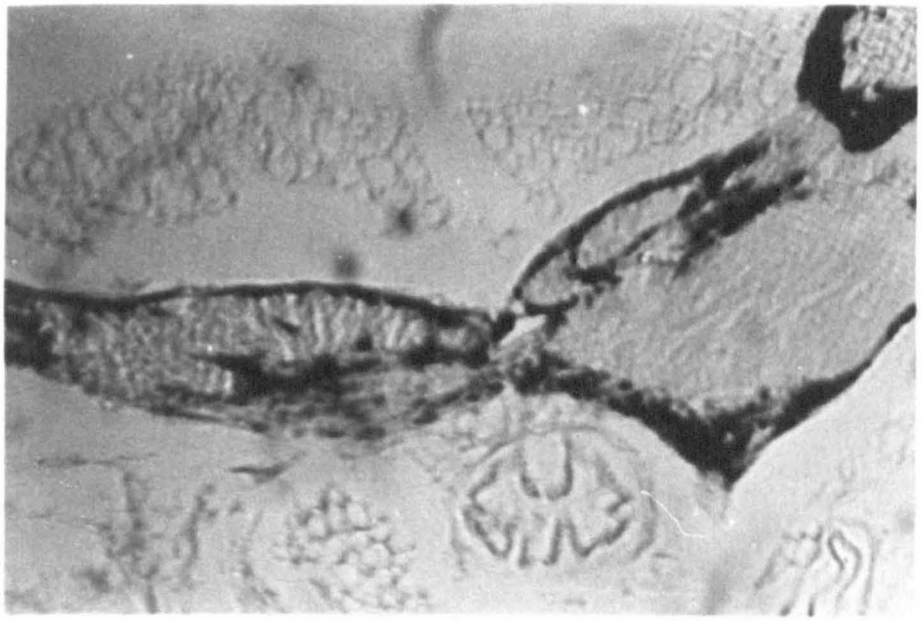


PLATE 14 (T2b) PROTOCEREBRUM x 100

PLATE 15 (T3) OPTIC LOBE x 100

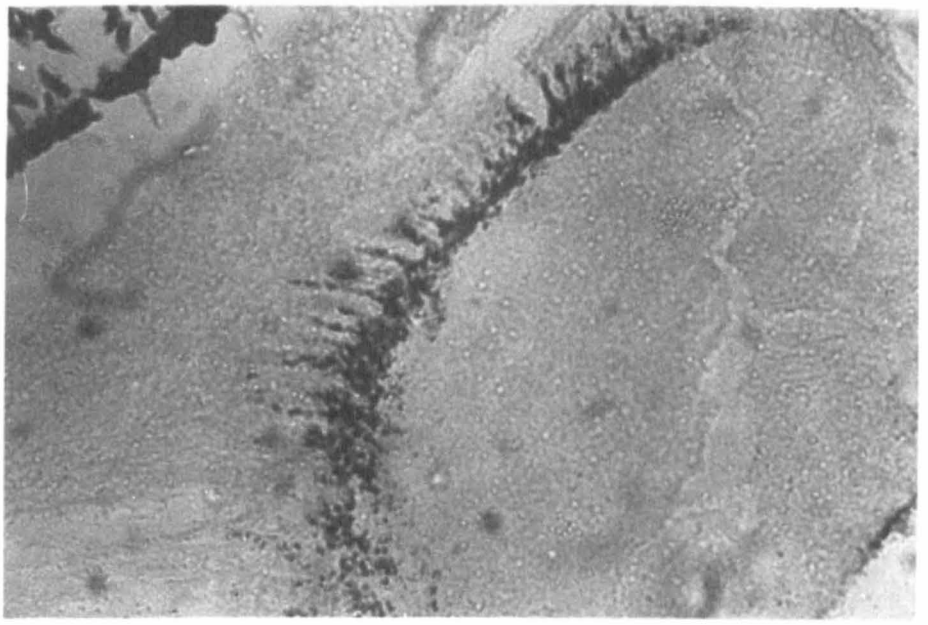


PLATE 16 (T3) OPTIC LOBE x 200

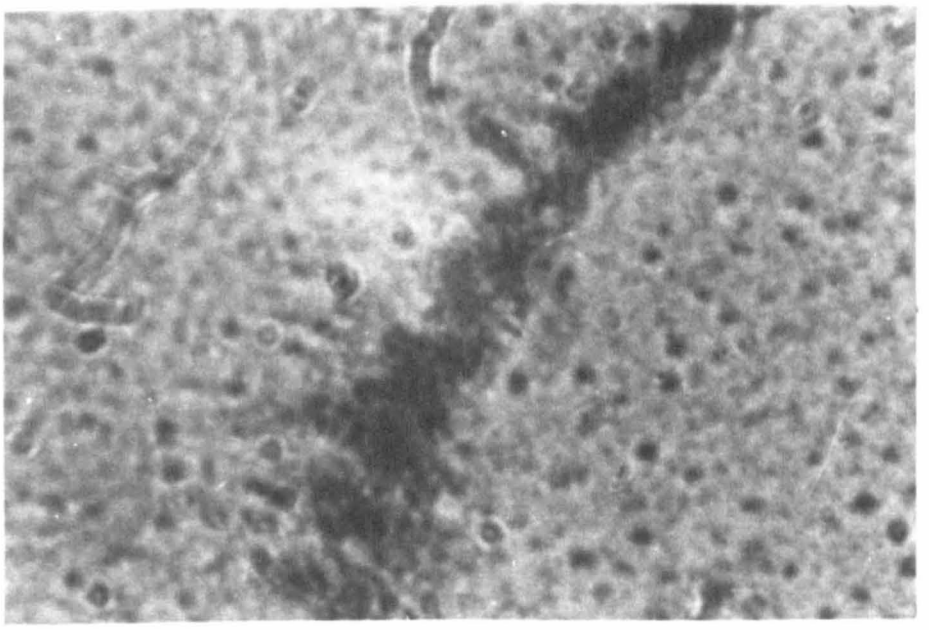


PLATE 17 (T3) OPTIC LOBE x 400

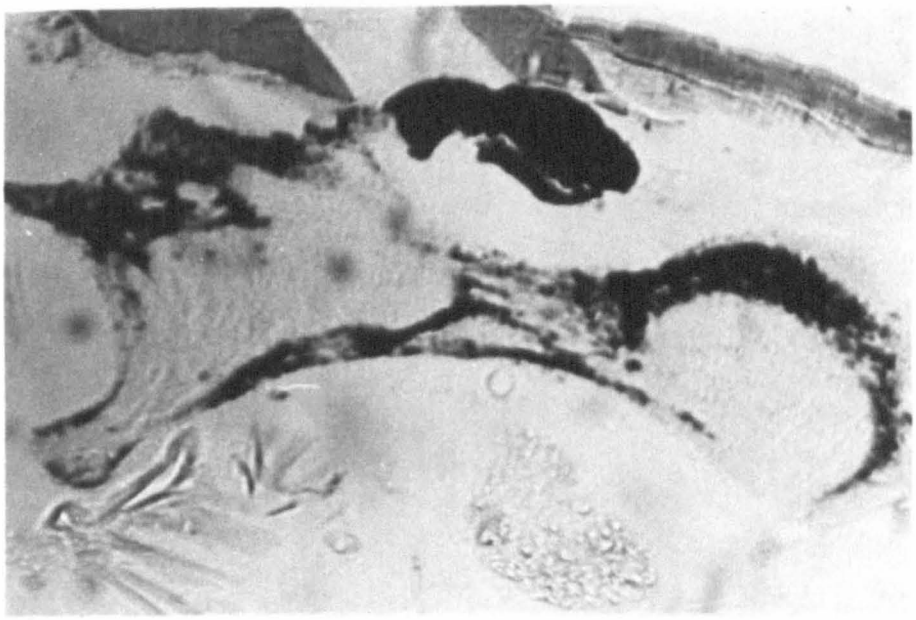


PLATE 18 (L1) BRAIN AND OPTIC LOBE x100

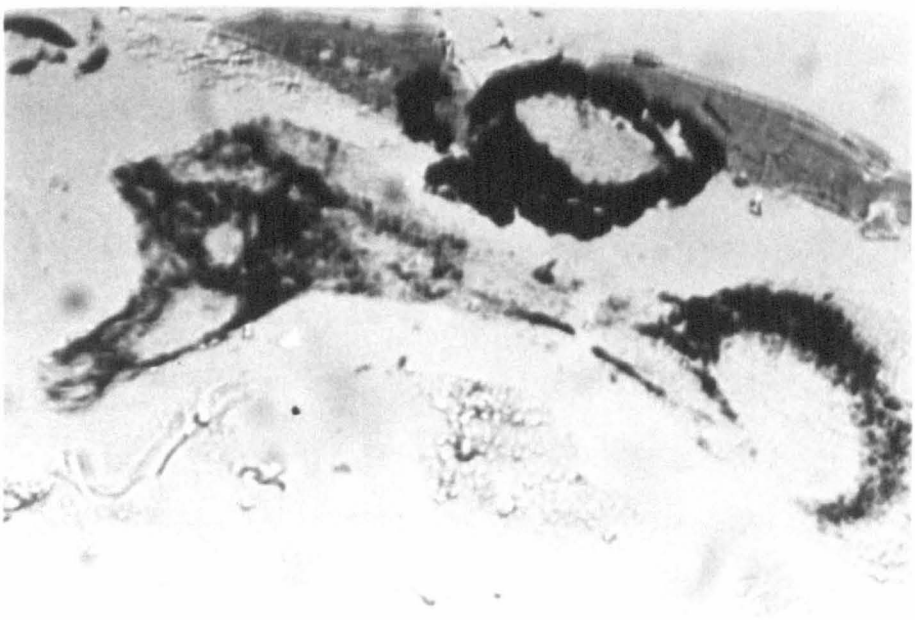


PLATE 19 (L2) BRAIN AND OPTIC LOBE x100

The function concerned with maintaining the general tonus is of interest because observations made on many nymphs during toxicity tests showed that the loss of characteristic body posture accompanies the onset of chronic toxicity. Further, the protocerebrum contains the so-called 'mushroom bodies' which are collections of association neurones. These are regarded as the most important centres regulating behaviour (WIGGLESWORTH,1967), and are highly developed in most insects. It is possible, therefore, that disruption of this area of the brain could lead to widespread effects on orientation generally. Lack of orientation may lead to a higher level of activity for a time, due to the reduced ability of the nymphs to become attached. This would result in a faster metabolic rate which could accentuate the effect of copper if it is a physiological one. If this is taking place it is, however, for a short time only, since observations made on the behaviour of nymphs during toxicity tests show that they actually become less active after several hours in dilute solutions of copper and after shorter periods of time in higher concentrations. It is possible, however, that there is a period in the early stages when activity levels are increased, and another factor that would contribute to this is the failure of the brain to exert its inhibitory influence on the sub-oesophageal ganglion. Observations of nymphs during toxicity tests seem to support this idea in that nymphs in copper solutions often lie on their backs making energetic movements with their legs. Thus both lack of orientation and unnecessary locomotory movements were seen to be one result of at least one stage of copper toxicity.

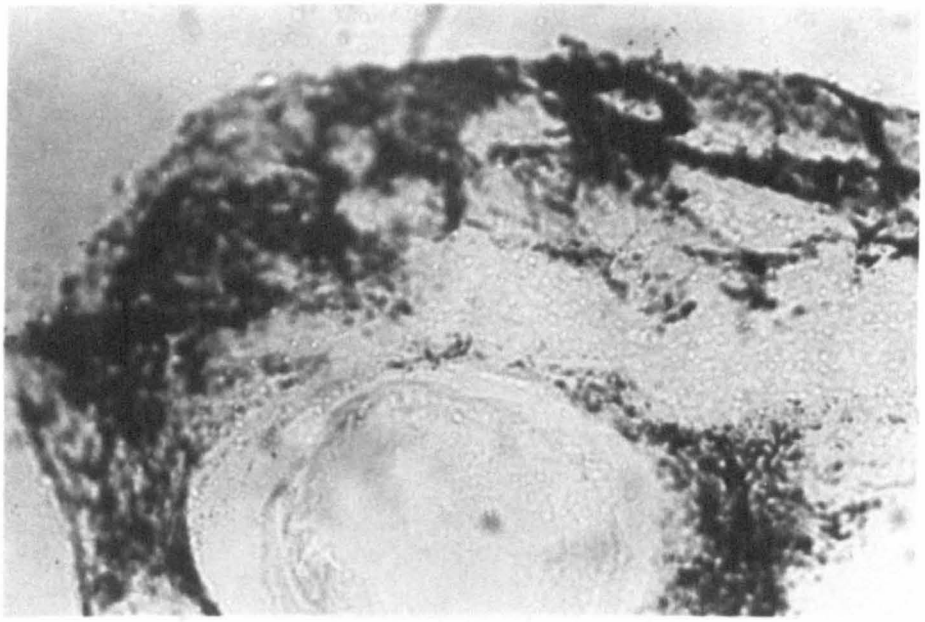


PLATE 20 (L3) PROTOCEREBRUM x200

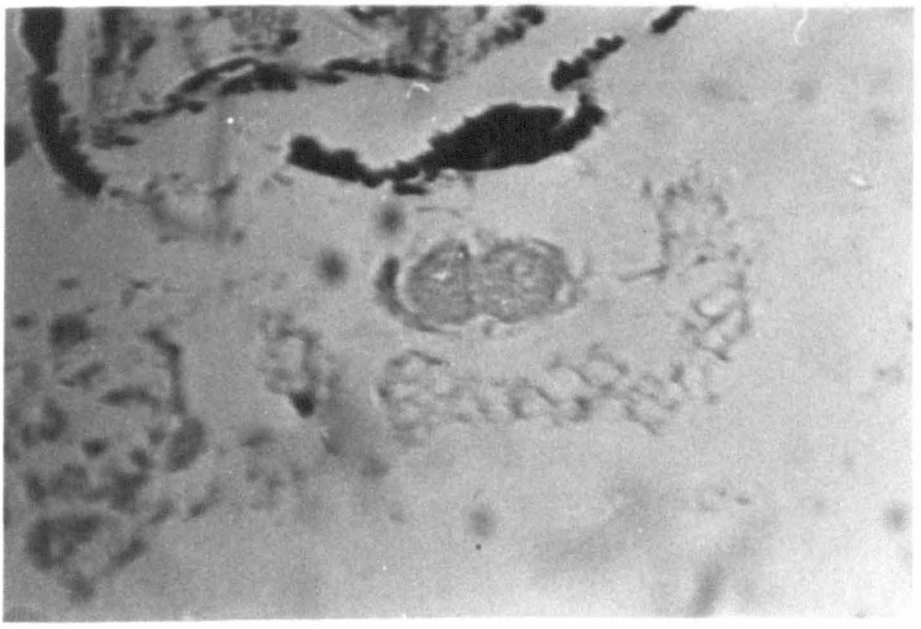


PLATE 21 (T4) VENTRAL NERVE CORD x100

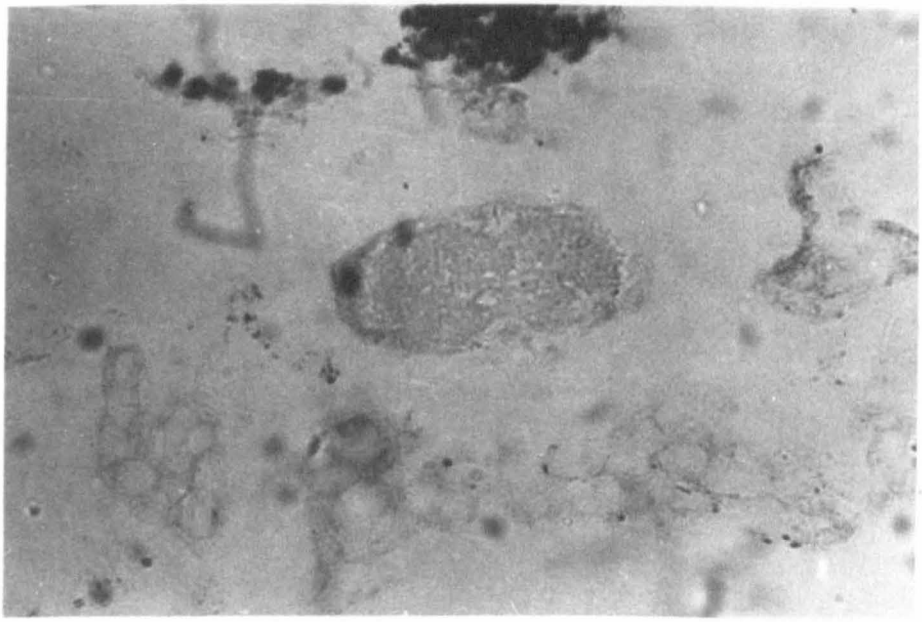


PLATE 22 (T5) VENTRAL NERVE CORD x200

Effects on the brain as such would not of themselves necessarily be a primary cause of death, but nymphs with impaired locomotive faculties would in nature obviously be unable to function normally in terms of escaping predators, feeding and maintaining their position in the stream. This evidence does, however, show one level of copper toxicity albeit a secondary one. It does not, however, account for the deaths of nymphs in the laboratory conditions which take place in what is, after all, a short space of time. Nor do effects on the brain account for the graded effect where higher concentrations cause faster rates of mortality. It seems that since the brain of insects is only responsible for sensory and certain locomotive functions, an effect of toxicity on this area cannot be seen to be responsible, directly, for mortality.

The other area of the nervous system which is seen to accumulate copper is the ventral nerve cord. This is a median chain of segmental ganglia lying beneath the alimentary canal. As can be seen, the accumulation here is small (see Plates 21 (T4) and 22 (T5)) and does not approach that found in the tissue of the brain. The significance of copper accumulation here is of some interest because the segmental ganglia are responsible for the control of respiratory movements which, in the case of mayfly nymphs, are concerned with the movement of the abdominal gills. The gill plate of E.venosus carries numerous sensillae which are sensitive to external factors and pass the information to the segmental ganglia which initiate an appropriate response. The movements of the gill plates are highly coordinated, their function being to bring about a current of water over the gill

filaments where exchange of gases takes place. In this way the filaments are supplied with a continual stream of fresh water and are thus able to exploit its dissolved oxygen content. In the case of E.venosus and R.semicolorata a disruption of this mechanism could reduce greatly the efficiency of the gill filaments as respiratory surfaces. This is not so much the case with B.rhodani since this species is found in waters in which the dissolved oxygen concentration rarely falls to below 4mg/l and so there is no need for accessory mechanical aid and the gills are non-motile (WINGFIELD,1937).

In the case of E.venosus (and possibly R.semicolorata), therefore, the implications of copper accumulations in the ventral nerve cord can be seen in terms of an interference with one aspect of respiration. This in itself may not be sufficient to bring about death but would contribute to a general weakening, making the nymphs less resistant to the effects of copper.

Other organs

The other areas of copper accumulation fall into the following regions:

a) the gill filaments

b) the malpighian tubules

c) the fat bodies

a) The gill filaments

The accumulation of copper in the gill filaments carries with it the obvious implications concerning respiration. As can be seen

from Plate 23 (T2), the accumulation is moderate generally but with fairly heavy accumulation in some filaments.

The gill filaments obtain oxygen that is dissolved in the water around them by diffusion. Interference at this point would, therefore, be due to some sort of physical barrier being set up which would prevent this diffusion of oxygen into the filaments. Whether this is unequivocally the case here is not possible to say from the visual evidence alone, but it is reasonable to expect some lowering in the general efficiency of each filament in its role of oxygen uptake. This lowering in efficiency may, of course, be very small in each individual filament, but could build up to a significant effect when multiplied by the drop in efficiency of all the filaments on the nymph. This accumulative effect may be considerable when one considers the number of gills involved. Each nymph had seven pairs of abdominal gills each with an average of twenty filaments. This gives a total of 280 filaments per nymph of E.venosus. Assuming that each tuft of filaments receives about equal amounts of copper, this builds up to a sizeable accumulation. This effect could be magnified when one adds to it that brought about by the reduced efficiency of the gill plates due to interference of the segmental ganglia of the ventral nerve cord. It seems possible, therefore, that copper could be having an effect on the respiration of the nymphs at two sites:

a) at the gill filaments themselves

b) at the ventral nerve cord

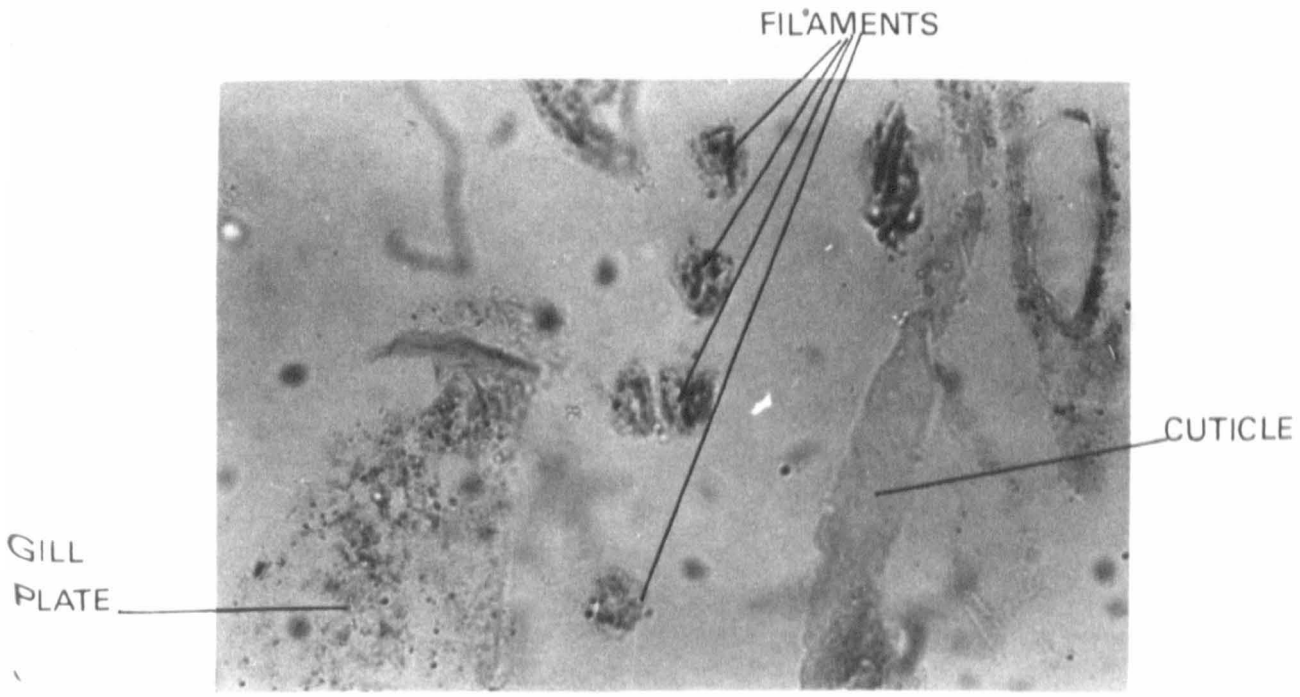


PLATE 23 (T2) GILL FILAMENTS x200

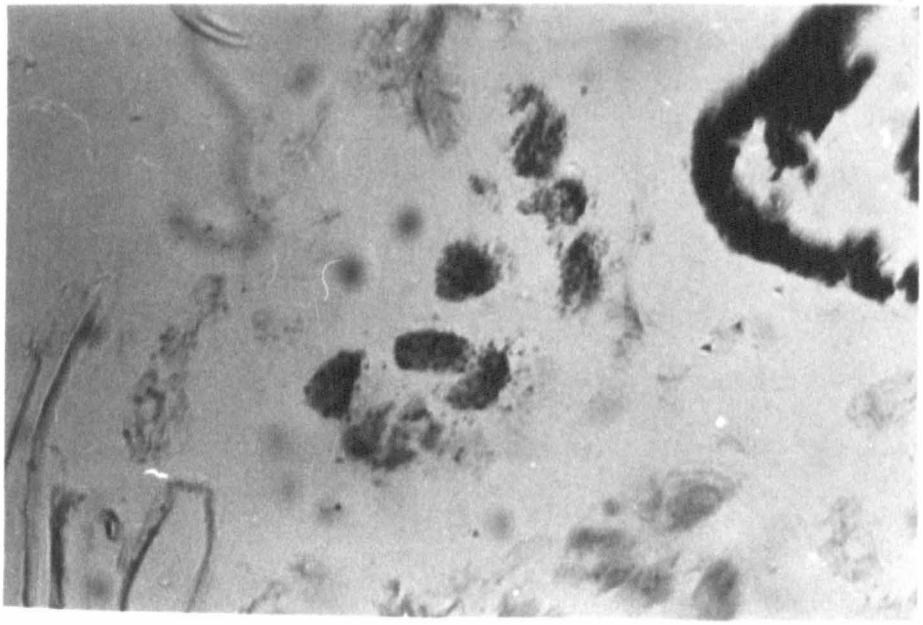


PLATE 24 (T1) MALPIGHIAN TUBULES x100

b) The Malpighian tubules (Plate 24 (T1))

In insects the Malpighian tubules are the organs of nitrogenous excretion. They are, therefore, responsible for the elimination of true metabolic waste products as opposed to the egestion of indigestible material. The presence of copper in these tubules is therefore interesting because it strongly suggests that copper is indeed travelling through metabolic pathways within the nymph. The actual extent of involvement of copper is not certain, however, since the Malpighian tubules are also responsible for the maintenance of a more or less constant internal environment and so will eliminate classes of substances other than those that can be termed nitrogenous. These substances include mineral salts or water which are present in excess in the diet as well as others which might upset the hydrogen ion balance in the body fluids or those which may interfere with certain osmotic relationships. From the picture obtained from the mid-gut region of the nymphs it seems likely that copper may fit into the class of substance constituting a simple excess of a particular ion, and can also be seen as a substance which in excess would also interfere with certain pH values and osmotic relationships. The presence of large amounts of copper in the haemocoel would therefore seem to call for elimination by the tubules.

However the copper is reaching the tubules its presence there does indicate that active excretion of the ion is taking place by the nymphs. This is interesting in itself because it could be an important factor to take into consideration in terms of 'safe' levels of copper concentrations. Presumably if the rate of uptake does not

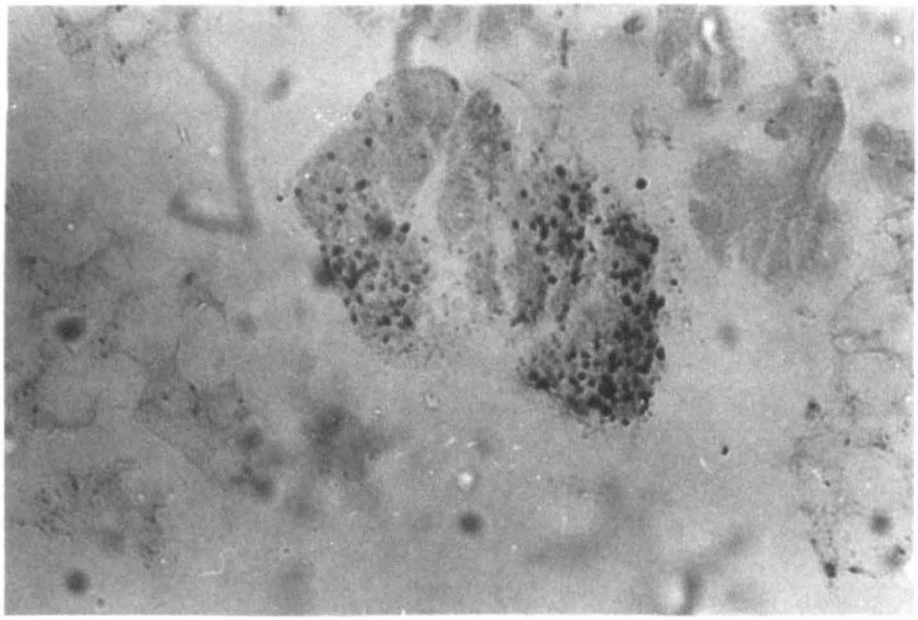


PLATE 25 (T3) FAT BODY x 200

exceed the rate of elimination the nymphs would be able to prevent accumulation occurring. This would have obvious effects on survival times. Also, presumably, the higher the external concentration, the faster does the rate of uptake become until the stage is reached where the excretory system can no longer eliminate sufficient quantities of copper and accumulation occurs. This could explain the results of the toxicity tests where higher concentrations resulted in shorter survival times.

c) The Fat body (Plate 25 (T3))

The fat body is found in all insects. It is found in the haemocoel and is therefore immersed in the blood which circulates through the interstices of the tissue. The main function of the fat body is linked to its position within the cavity of the haemocoel and this is to separate from the blood reserve materials and to act as a storage tissue for these materials. As can be seen here, copper also appears to be taken up and 'stored' by this tissue. This is an indication that the amount of internal copper is in excess of amounts which could be dealt with by the malpighian tubules.

B.rhodani and R.semicolorata

Rubeanic acid preparations made with the other two species used in this study showed very similar internal copper distributions. Plates 26 - 29 are typical examples.

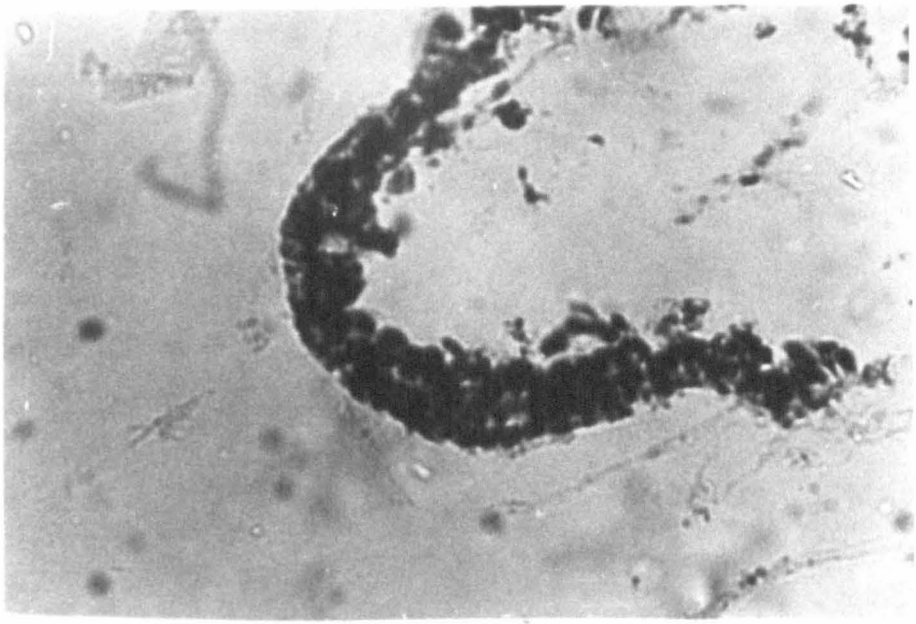


PLATE 26 B.rodani TS mid gut x 200

PLATE 27 B.rhodani LS brain x 100

plate 28 R.semicolorata L.S. brain and optic lobe x 100

plate 29 R.semicolorata T.S. gill filaments x 100

NYMPHS NOT EXPOSED TO COPPER

Sections prepared from nymphs not exposed to copper solution were also treated with rubeanic acid. In this case no area within the body produced the black staining characteristic of the presence of copper, except for the ommatidia of the compound eyes. This, however, was not due to the presence of copper but to the naturally occurring pigment in these structures. The same black coloration appeared in sections not treated with rubeanic acid.

SUMMARY

1. Rubeanic acid has been used to detect copper in sections of nymphs exposed to copper containing solutions.

2. This technique has demonstrated that copper is being taken up and accumulated within certain tissues of the nymphs.

3. The tissues where copper accumulation occurs are in the following regions:

    a) the alimentary canal
    b) the central nervous system
    c) other organs: the malpighian tubules, the gill filaments, and the fat body.

    In these tissues the accumulation appears to be intracellular.

4. No evidence was found to show that copper is being adsorbed on any external surfaces although this finding must be qualified by the possibility of any adsorbed being lost during fixing and staining processes.

5. The distribution of internal copper is the same in all the species studied.

6. The theoretical implications of these distributions are discussed.

Chapter 7

EFFECT OF COPPER ON RESPIRATION

INTRODUCTION

In 1926 a study was made on the effect of heavy metals, including copper, on the respiration of the fungus Aspergillus niger (COOK,1926). Here it was found that an initial period existed during which there seemed to be no effect on respiration. This was then followed by a sudden and dramatic drop in oxygen consumption. Another study demonstrated that in fish, solutions of heavy metals produced an initial increase in respiration followed by a decline in respiration rate (JONES,1946).

Apart from these two studies there appears to have been very little work carried out into the effect of copper on respiration. In the more specific case of aquatic insects there seems to be no information available. This study was therefore carried out partly to remedy this situation to a small extent.

In the previous chapter it was demonstrated that copper is entering the nymphs, and so it is reasonable to look for some internal, physiological effects and hope to find some indication of the type of action involved.

Respiration is a process basic to the general metabolism of an organism and therefore would seem to be a good starting point for work which is trying to lead to some explanations concerning the action of copper in an organism. Another reason for considering this a useful starting point was supplied by evidence from Chapter Six which showed that copper was present in the gill filaments, which suggests some action here. Other sites, also, are possible but

respiration provides a relatively easily measurable process when looked at in terms of oxygen consumption.

For these reasons it was, therefore, decided that an investigation into the effect of copper on the respiration rates of the three species of mayfly nymph used in this study should be the next step.

MATERIALS AND METHODS

In choosing a method for measuring the respiration of the nymphs certain conditions had to be satisfied. These were mainly concerned with providing an environment as similar as possible to the natural so that the respiration rates measured were not subject to too many variables. This meant that, taking the ecology of the nymphs into consideration, certain methods which have become almost standard in the past for the measurement of respiration in aquatic invertebrates could not be employed here. These methods fall into several categories:

1. 'Closed-bottle' techniques where the disappearance of oxygen in a closed bottle containing a known volume of water is taken to represent a measure of the oxygen consumed by the organisms being investigated. The main objection against this method is concerned with the respiration of mayfly nymphs. As already pointed out in an earlier chapter, the respiration of the nymphs of mayflies is closely related to their physical environment. The three species used in this study are all found in running waters and the respiratory movements of their gills is extremely sensitive to this factor, so that a current is necessary for the natural functioning of the gills. The closed-bottle technique, therefore, in which the nymphs would be tested in still water, would present a totally unnatural set of conditions against which to attempt to make measurements of oxygen uptake. Closed-bottle techniques involving still water were, therefore, considered unsuitable for the purpose of this study.

2. Warburg apparatus. Here the nymphs would also be confined in a closed bottle but the water would be subjected to continuous agitation. This, however, is also unsuitable because the important point about the water movement which is required for these nymphs is that it be unidirectional. The Warburg apparatus would not only bring about agitation unsuitable for normal respiration but would also affect the normal activity of the nymphs, and thus bring about another abnormal factor which would also contribute towards an effect on respiration. This would be mainly due to the fact that in water producing random movement, as opposed to a unidirectional flow, the nymphs would not be able to orientate themselves normally, which is a necessary requirement to settling. This could produce higher than normal activity.

The Warburg technique was tried out and these trials showed up another factor which made this technique unsuitable. It was found that, because of the small volume of liquid used in each vessel, the carbon dioxide produced by the nymphs was causing precipitation of copper, presumably in the form of carbonate, thus making it impossible to test the effect of different concentrations of copper in solution.

3. There are other methods which do employ a continuous unidirectional flow of water, but here the flow is usually very slow and nothing like that found in the natural habitat.

Bearing these objections to the methods usually in mind, several points emerge which seem to be necessary in choosing a method which could be considered suitable. These are:

1. The maintenance of a unidirectional flow of water.
2. That this flow is of a velocity that approaches at least that found in the natural habitat of the nymphs.
3. That the volume of water is sufficiently large so that evolved carbon dioxide does not interfere appreciably with the dissolved copper salt.

A fourth point might be added here which does not directly come into the above but is of some importance. This is that the sensitivity of the apparatus is sufficient to detect changes taking place in necessarily large volumes of water.

The apparatus which seems to satisfy all these points is based on a manometric method of measuring oxygen uptake. It consists of two parts:

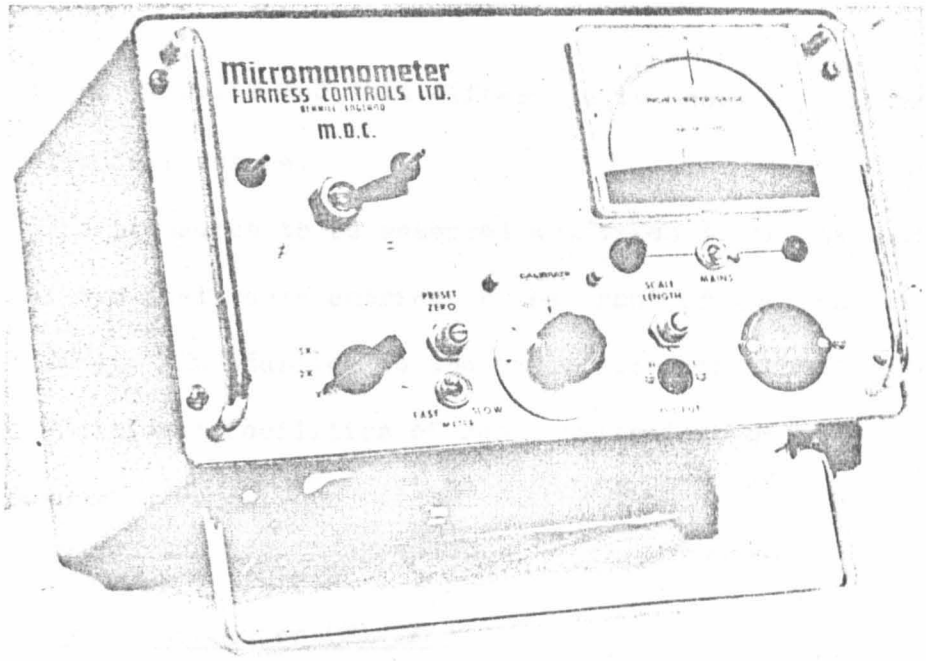
1. A micromanometer
2. A respirometer

1. <u>The micromanometer (Plate 30)</u>

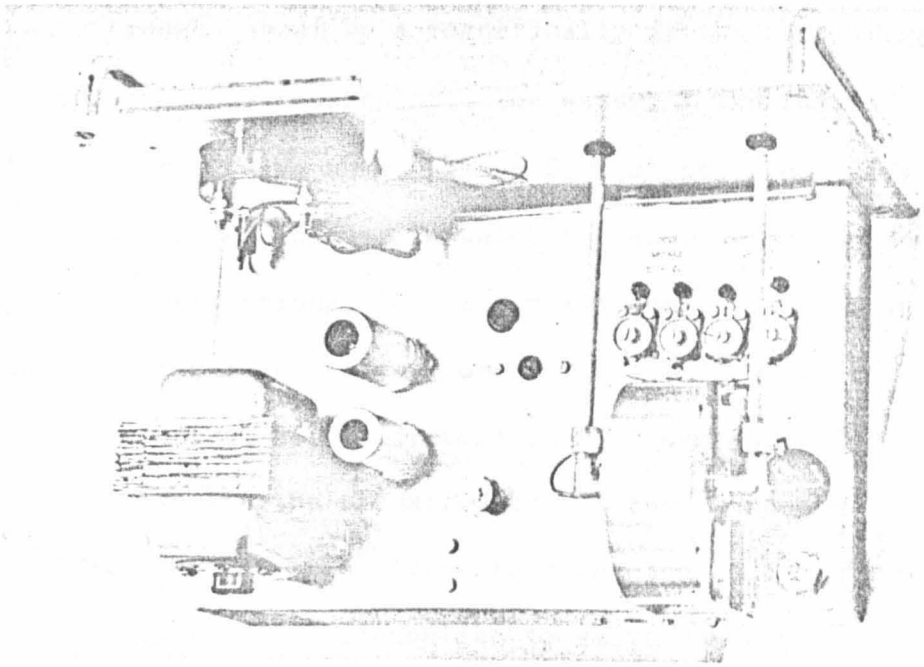
This instrument was supplied by Furness Controls Ltd. It is highly sensitive and capable of measuring differential gas pressures down to 10 dynes/sq.cm. and converting these to readings on a linear scale.

The measuring consists of two symmetrically arranged cavities separated by a metal diaphragm. The diaphragm, together with a fixed electrode on either side, forms two condensers which are part of the tuning capacities of two tuned circuits. Movement of the diaphragm causes a variation in the capacitance between it and each adjacent electrode. This, in turn, unbalances the voltage across

PLATE 30 THE MICROMANOMOMETER



FRONT PANEL



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the tuned circuits and this difference is shown on a meter calibrated directly in pressure.

The pressures to be measured are taken to the measuring head by way of two small hose entries on the front of the instrument (see Plate 30). In addition to the basic circuits the micromanometer has also additional facilities of range switching, giving 2X and 5X increases in range.

2. The respirometer (Fig.27)

This is constructed of perspex and wide (1-inch diameter) PVC opaque grey tubing. It provides a unidirectional flow of water which is brought about by a magnetically driven centrifugal pump which continuously recirculates the water. The rate of flow is controlled by a valve which allows for selection of different rates. There is a perspex chamber in which the animals are housed during experiments (dimensions, 30cm x 4cm x 3.6cm). This chamber is fitted with two mesh screens, one for the inflow and the other for the outflow apertures, to prevent loss of nymphs. To this chamber are fitted two lengths of narrow-bore tubes (connected by airtight fitments). One of these tubes is connected to the micromanometer, while the other is for connection to valve the closure of which isolates the chamber from the atmosphere.

The remainder of the apparatus consists of a compensation vessel of similar dimensions to the chamber described above. This also has similar tubes attached to it and is closed to the atmosphere at the same time as the respirometer chamber at the start of each experiment.

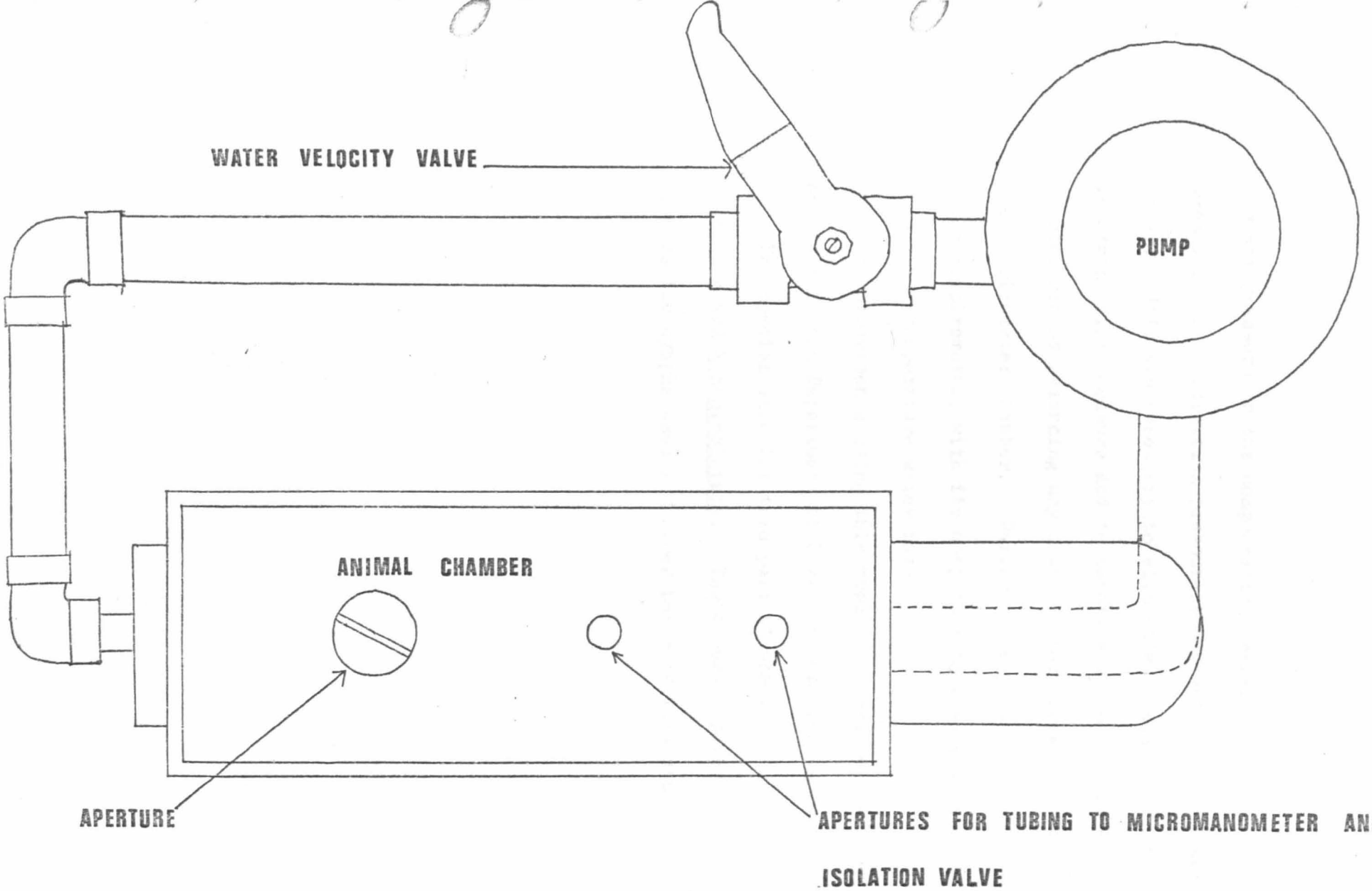


FIG 27 THE RESPIROMETER

The air pressure in the compensation vessel acts as a reference pressure for differential pressure measurements by the micromanometer. This was necessary to eliminate errors due to fluctuations in atmospheric pressure and temperature which the compensation chamber did by balancing any changes that these factors caused in the respirometer chamber. During the course of experiments the whole respirometer, with its compensating chamber, was submerged in a constant temperature water bath.

The experiments using this apparatus were carried out in a basement of the Department of Civil Engineering building.

The species used for this part of the study were B.rhodani, E.venosus and R.semicolorata. These were collected from the same site as the nymphs used for other parts of this study.

PROCEDURE

Depending on the size of nymphs available, ten to twenty were used in each experiment. All the nymphs used were starved for a period of about twelve hours before being used in experiments. This was done to ensure some degree of uniformity as regards the metabolic state of the nymphs, making comparisons between different experiments more meaningful. Only nymphs that appeared to show normal activity were used in these experiments.

At the beginning of each experiment the respirometer was partially filled with a copper sulphate solution which had been aerated for a period of not less than one hour. The oxygen concentration of this was determined using the Winkler method and solutions of as uniform dissolved oxygen content as possible were used. The solution was poured into the respirometer through a $\frac{3}{4}$-inch diameter aperture at the top of the perspex chamber. The nymphs, contained in a copper solution of the same concentration as that in the respirometer, were introduced into the chamber through the same aperture. The respirometer was then completely filled, some care being taken to exclude air bubbles. The amount of solution placed into the apparatus was $500cm^2$ and from this exactly $100cm^2$ were removed, using a $100cm^2$ volumetric pipette, so that a gas space of known volume was always present throughout this series of experiments (this was subject to a slight error due to displacement caused by the nymphs). Some potassium hydroxide solution soaked onto a piece of filter paper for greater surface area was introduced into the apparatus by means of a well designed for this purpose that

was built into the respirometer. The aperture through which the nymphs were introduced was sealed by means of a plastic screw thread stopper. This was smeared with a silicone grease to ensure an airtight seal. Other joints were also smeared with silicone grease. The whole apparatus was then submerged completely in the water bath, the pump switched on and the flow valve adjusted to give what was judged to be an appropriate water velocity. Information regarding this had previously been obtained by using a current meter at the collecting site on the River Coquet. The temperature of these experiments was $12^{o}C \pm 1^{o}C$.

Ideally, some time should have been given for acclimatization before taking recordings but this was not possible since the nymphs were in a toxic solution and any early effects would have been missed. A continuous record of the pressure changes in the respirometer was made in all the experiments by using a servoscribe chart recorder connected to the micromanometer. Immediately before recording the valves connecting the gas spaces were closed at the same time.

Each experiment lasted approximately eleven hours and three experiments were carried out at each concentration of copper sulphate used. At the end of each experiment the nymphs were killed and dried in an oven at $100^{o}C$ for twelve hours. They were then weighed on an electronic balance. The results were therefore calculated as consumption of mg oxygen per gm dry weight per hour.

Using the traces obtained from the chart recorder, the rate of total oxygen consumption per hour was calculated using the following equation:

$$x = h \left[\frac{VG \frac{273}{T} + VF \alpha}{Po} \right]$$

where:

x = amount of oxygen consumed, mm^3 at NTP
h = micromanometer reading, mm water gauge
VG = volume of gas space in respirometer
T = temperature of water bath, ^oA
VF = volume of liquid in respirometer
α = solubility of evolved gas at T^o
Po = normal pressure in mm, water gauge

For each group of three experiments at a particular concentration of copper sulphate the standard error was calculated. This is expressed as + or - value after each result in the tables of results.

It must be noted here that the results obtained represent the <u>total</u> amount of oxygen consumed by <u>all the organisms present</u> in the respirometer, i.e. they include oxygen consumed by micro-organisms which were probably introduced into the respirometer with the nymphs. Also, some initial problems were experienced with leaks in various parts of the apparatus. These were overcome, however, with liberal applications of silicone grease. Some incalculable degree of error originating from this factor probably persisted, however.

DURATION OF EXPERIMENTS (100 and 200mg/l Copper Sulphate)

It was felt that it would be desirable to prolong the experiments for a reasonable length of time so that there would be a greater chance to detect any effects of copper on respiration which might not necessarily be immediate. This presented obvious difficulties with the experiments involving the higher concentrations of copper, since the nymphs would start to die in a relatively short time from the start of the experiment. From earlier experiments it was found that the three species had the following Tlms (approximately):

species	concentration copper sulphate mg/l	
	100	200
B.rhodani	6.8	6.4
E.venosus	8.0	6.4
R.semicolorata	7.8	6.3
	(times in hours)	

This meant that, at the times shown above, half the animals would be dead. This presented the problem of how to account for this in an experiment lasting over ten hours. To try to overcome this problem the following procedure was adopted in the experiments involving 100 and 200mg/l copper sulphate (it was also used in some of the other experiments when necessary, and in the case of B.rhodani it was used at 50mg/l copper sulphate).

A very close watch was made on the nymphs during the experiment. This involved turning the water velocity down to a low level for a period of about one to two minutes while observations were being made. Through the perspex lid of the respirometer (animal) chamber it was quite easy to see which nymphs had died and the times of death were noted. The water velocity was then restored to its original speed and the experiment allowed tocontinue with the surviving nymphs. At the end of the experiment surviving nymphs were killed and treated as described above. The dead nymphs were also dried and weighed and the weight added to that of the surviving nymphs, giving the total dry weight for all the nymphs. In the subsequent calculations an assumption had to be made that the nymphs weighed approximately the same (for this reason nymphs of as uniform size as possible were used in these experiments), so that with the times of mortalities it was possible to make appropriate allowances for these deaths.

This procedure obviously introduces another source of error which comes into effect and becomes greater after about six hours. It was felt worth using this method, however, to gain additional information so that comparisons could be made with other concentrations.

RESULTS

In looking at the results obtained from this set of experiments it would be best if each species is looked at in turn before attempting detailed explanations and comparisons.

In each case a table is given showing the oxygen consumption/minute/gm for each species. The data obtained represents the means of three experiments and which are followed by the standard error expressed as + or - values. The curves obtained from this data are also shown for each species of nymph at the concentrations of copper used.

Before looking at the individual results there are some general comments which ought to be made.

The first point to note is that at most of the concentrations there is an initial period lasting about 200 minutes which seems to be characterized by large fluctuations in consumption resulting in high standard error values. This is by no means always the case but is generally true. In interpreting these graphs, therefore, this initial period will be regarded as a 'settling down' phase in which, for varied reasons, the nymphs are respiring somewhat erratically. This could be due to such factors as the physical handling of the nymphs while introducing them into the apparatus, and also the attempts of the nymphs finding points of attachment in a new environment. Both of these factors could subject to large individual discrepancies in the behaviour of nymphs or groups of nymphs. For these reasons, therefore, the most significant or useful parts of the curves are taken to be those coming after approximately 150-200 minutes.

A second point which requires comment is the fact that even in the experiments in which no copper was present there is seen to be a gradual decline in respiratory rate. This has two possible explanations:

1. It could be due to a residual and diminishing 'settling down' effect in which the nymphs become increasingly more settled (i.e. attached), activity becomes less, this leading to a reduced oxygen requirement.

2. The apparatus is a closed system, so that there is a finite amount of oxygen available to the nymphs in any one experiment. As this becomes gradually depleted a reduced overall consumption could result.

A third possible reason could be argued, this due to the death of nymphs during the experiment. This possibility, however, was eliminated by keeping a very careful count at short intervals during the experiment so that deaths could be monitored. Data from experiments during which nymph mortalities occurred were treated in two ways:

1) If only one or two per cent nymphs died the time of death was noted and this was taken into account in subsequent calculations involving weights of nymphs and oxygen consumption.

2) If, for one reason or another, more than two per cent nymphs died, the data for that experiment was scrapped and a new experiment using fresh nymphs was set up.

In interpreting the curves, it is the <u>extent</u> or rate of the falling-off in oxygen consumption which is significant rather than

the actual decrease as such. This is especially important in making comparisons. Also, the oxygen consumption at the end of the experimental period (about 660 minutes) is perhaps a good point for making comparisons between the effects of different concentrations since this will be determined by the rate of falling off in consumption.

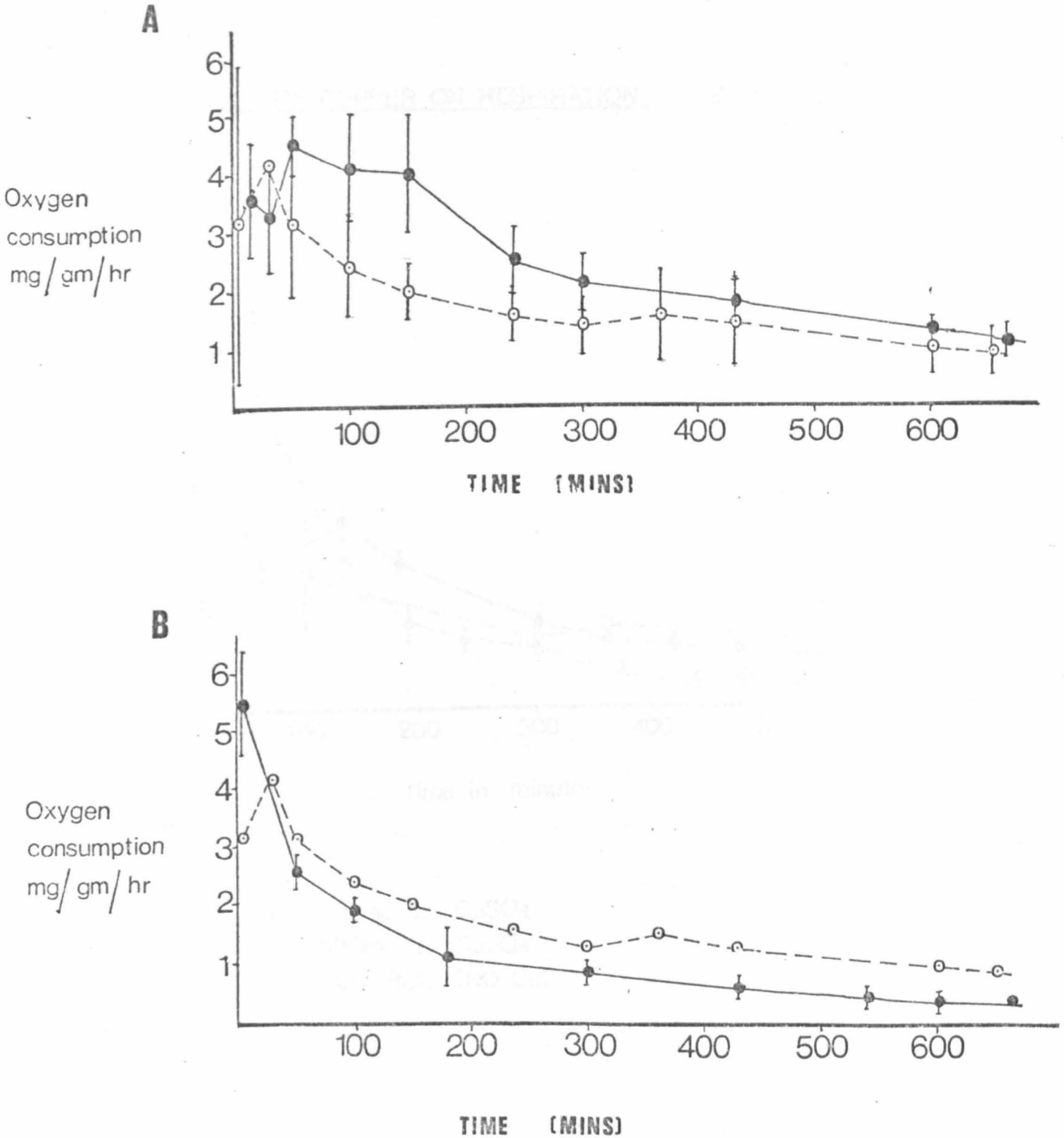
Ecdyonurus venosus

The table given in the appendix gives the data obtained for concentrations of 0, 10, 20, 50 and 100mg/l copper sulphate. Figs 28 to 29 show the graphs obtained from this data.

As can be seen from a comparison between the curve obtained for no copper with that for 10mg/l copper sulphate, at this concentration there does not appear to be any great effect on the respiratory rate of the nymphs. Generally, the O_2 consumption rate is higher, suggesting a stimulated rate for a short period with low concentration of copper.

The curve obtained for 20mg/l copper sulphate is difficult to explain when one considers those obtained for higher concentrations, for it is seen to be similar to that produced for 100mg/l copper sulphate. It is possible that a concentration of around 20mg/l is a threshold at which the concentration of copper is sufficiently high to affect the respiratory rate, concentrations above this making little difference. This point will be discussed later in relation to the results for the other two species.

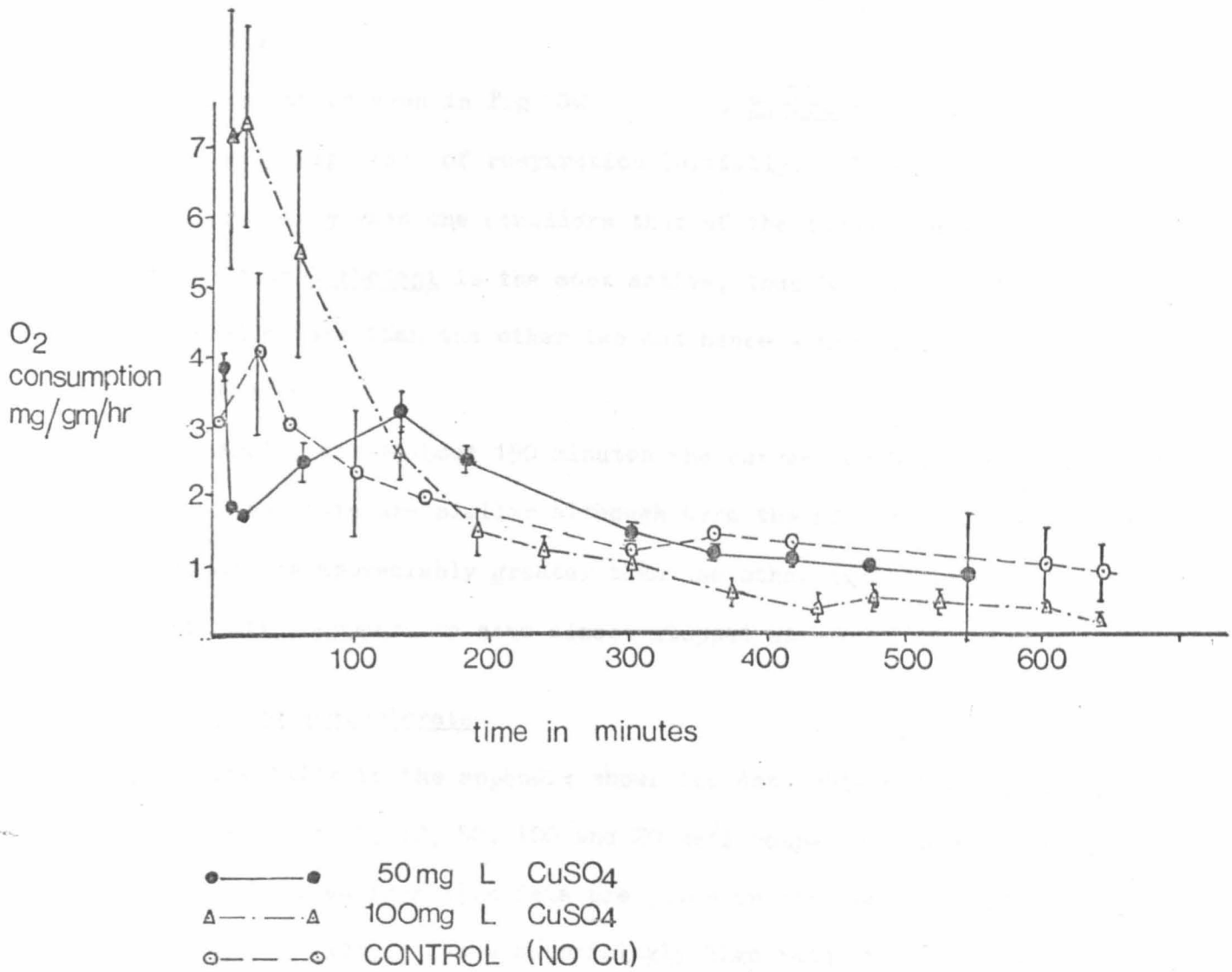
fig 28 effect of copper on respiration E.venosus



A ●——● 10mg/L $CuSO_4$; ⊙- - -⊙ CONTROL

B ●——● 20mg L $CuSO_4$; ⊙- - -⊙ CONTROL

FIG 29 EFFECT OF COPPER ON RESPIRATION. E.venosus



Baetis rhodani

Table 15 shows the data obtained from experiments using 0, 50, 100 and 200mg/l copper sulphate, and this will be found in the appendix.

As can be seen in Fig 30 , B.rhodani displays a generally high rate of respiration initially. This perhaps is not so surprising when one considers that of the three species used in this study B.rhodani is the most active, thus having a faster metabolic rate than the other two and hence a higher rate of respiration.

Again, after about 150 minutes the curves for 50, 100 and 200mg/l copper sulphate are similar although here the effect of 200mg/l copper sulphate is appreciably greater than the other two, to the extent that respiration appears to have almost stopped at around 660 minutes.

Rithrogena semicolorata

The table in the appendix shows the data obtained using the concentrations 0, 10, 50, 100 and 200mg/l copper sulphate. The graphs obtained from this data are given in Figs31a to b .

R.semicolorata has a surprisingly high rate of respiration in terms of a relationship between respiration and activity since this species is not generally thought to be as active in nature as B.rhodani. It is possible that in the experimental apparatus the nymphs did not become attached as successfully as E.venosus and so had a higher than normal activity level imposed on them by the artificial environment in which they found themselves.

In R.semicolorata, as in E.venosus, a concentration of 10mg/l

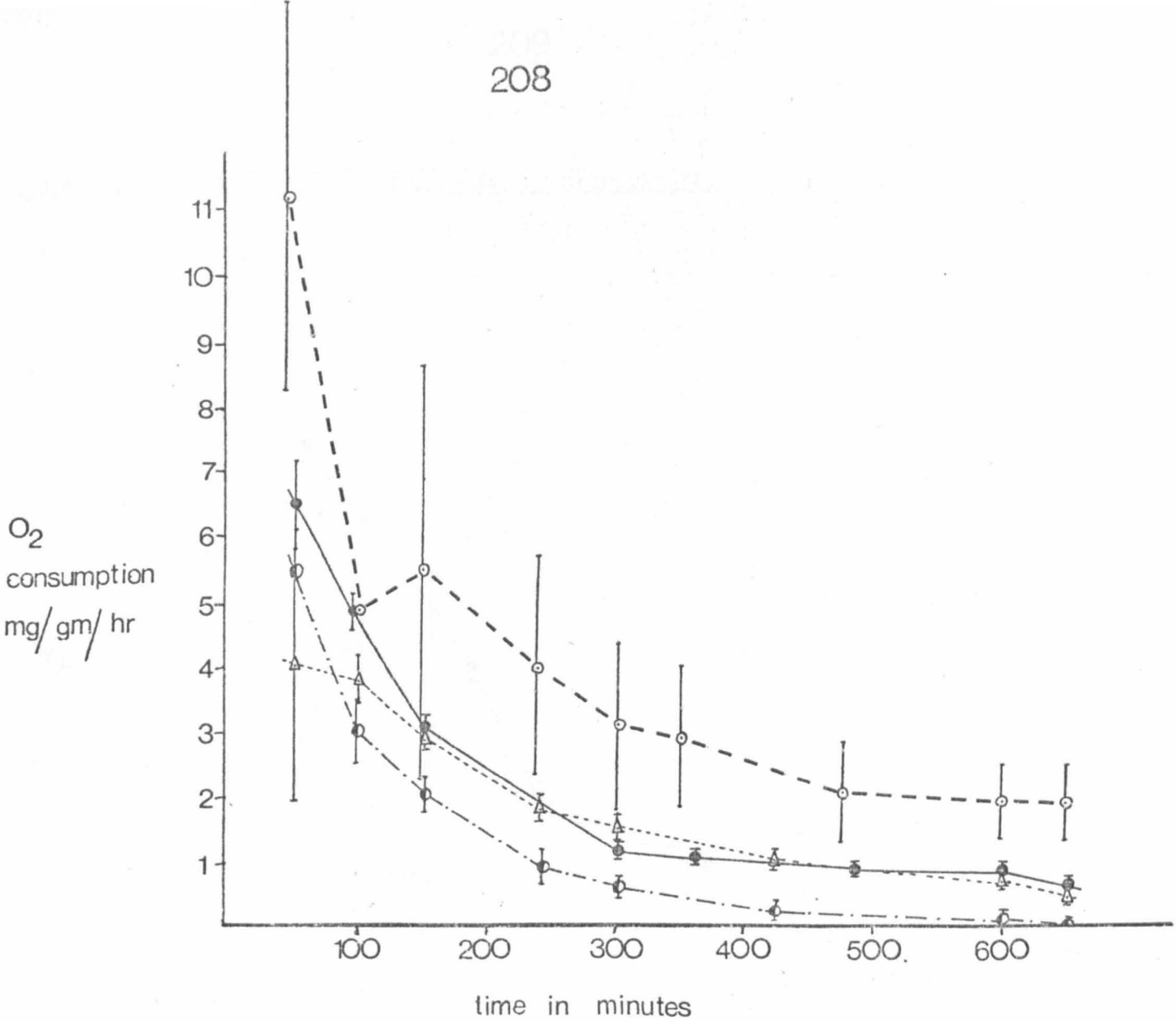
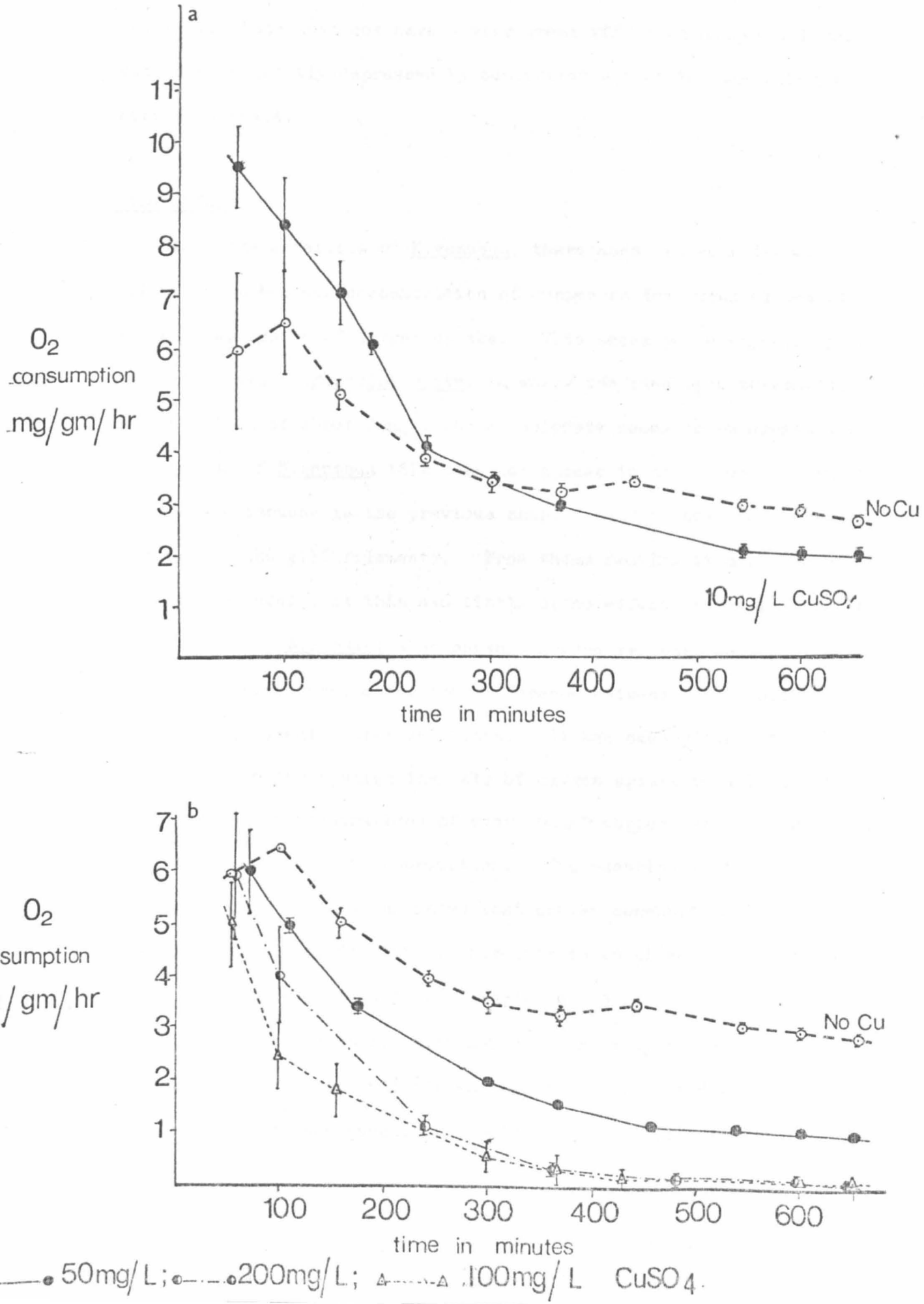


FIG 30 EFFECT OF COPPER ON RESPIRATION ; B.rhodani

●——● 50mg L $CuSO_4$
△-------△ 100mg L $CuSO_4$
●-·-·-● 200mg L $CuSO_4$
⊙ – – – ⊙ CONTROL (NO COPPER)

FIG 31 EFFECT OF COPPER ON RESPIRATION - R. semicolorata



copper sulphate does not have a very great effect on oxygen uptake, but this is greatly depressed by concentrations of 100 and 200mg/l copper sulphate.

DISCUSSION

With the exception of E.venosus, there seems to be a direct relationship between concentration of copper in the external medium and the depression of oxygen uptake. This seems to be especially so in the case of R.semicolorata, in which the idea of a threshold concentration of about 20mg/l copper sulphate seems to be operating. In the case of E.venosus this does not appear to hold true. This is interesting because in the previous chapter it was shown that copper is found in the gill filaments. From these results it must therefore be concluded that this has little or no effect on respiration in this species. B.rhodani also shows a graded response to a certain extent, although here, also, the difference between oxygen uptake is slight in different copper solutions. It has been shown, however, that in two of the species the rate of oxygen uptake is affected by copper in that concentrations of over 20mg/l copper sulphate bring about a definite drop in consumption. The question which these findings prompt is: having shown that oxygen consumption falls off in copper solutions, is this due directly to an effect which copper has on the respiratory mechanism itself, or is it an indirect effect due to some other metabolic site being affected by the copper, thus causing respiration to fall off as a secondary or incidental effect? This leads to another question: could the effect be a physical one

in which the gills are affected in a way similar to that in fish, thus causing a barrier which would effectively reduce the efficiency of the gills as mechanical respiratory surfaces? This would seem to be unlikely, since in the case of fish the copper had to combine with the mucin produced on the gill surface to form an impermeable barrier. There is no evidence to show that the gills of aquatic insects produce such a secretion. Surface effects as such can be argued against generally in the light of the findings of the previous chapter, where no evidence was found for copper adsorption on either the body or gill surfaces. The histochemistry did, however, show that copper does enter the body of the nymphs via the mouth, presumably when feeding and active drinking take place. Further, the copper was found to have been deposited inside the cells of the gut wall as well as being present within the lumen of the gut. In other words, it looks as if absorption is taking place. In view of this it is easier to conceive of copper as having a true internal, physiological effect.

Of the mechanisms which could be disrupted it is difficult to think of one which does not in some way have an influence on respiration in some way. It could be argued that the cells of the gut are affected in some way so as to prevent them absorbing products of digestion. If this is so, then it is unlikely that the effects of this would manifest themselves as quickly as to produce results within the relatively short period of the experiments. Also, there is the fact that the nymphs were starved for 12 hours before they were used in experiments in an attempt to ensure a reasonably equal

metabolic condition in the nymphs used in experiments. By the time the nymphs were used, therefore, they should have been utilizing already absorbed and assimilated reserves, mostly in the form of glycogen. It could be argued that copper in some way affects this process but again the time scale involved is too short for this to show up in the course of the experiments. The same arguments apply to the possibility of copper interfering with digestive enzymes. It seems, therefore, that interference with the digestive physiology of the nymphs is unlikely.

In the previous chapter it was demonstrated that copper is present in the nervous tissues, particularly in the brain where it was found accumulated in the protocerebrum and parts of the optic lobes. Also copper was found in the ventral nerve cord. This is additional evidence that it is being absorbed through the gut wall to be distributed and accumulated in various tissues of the body. The fact that this tissue is nervous tissue is interesting for two reasons:

1. The accumulations of large amounts of copper in such vital tissue as that comprising the central nervous system could be having far-reaching consequences for many aspects of the nymph's normal functioning which come under the control of the central nervous system.

2. The respiratory movements of insects (in the case of aquatic insects movements of the gills) are controlled by impulses from nerve centres. These nerve centres are situated in the so-called primary respiratory centres which lie in the segmental ganglia and control

the movements in their own segments (WIGGLESWORTH,1966). The ventral nerve cord is, in fact, a median chain made up of segmental ganglia lying just beneath the alimentary canal. It is possible that the accumulation of copper in the tissue of the segmental ganglia could in some way be interfering with their roles of respiratory centres. In this way copper could be affecting mechanical or external respiration by preventing the gills functioning normally.

Another tissue which is associated with respiration is the blood or haemolymph. This is the only tissue fluid found in insects and is thus solely responsible for the transport of substances within the body. As we have seen that copper is transported in the body, the blood must be coming into close contact with it. It has been established that although oxygen is ordinarily conveyed directly to the tissues by the tracheal system, in many circumstances the blood also acts as an oxygen carrier. It is possible, therefore, that copper is exerting some influence at this level also. Also, it is possible that copper could be exerting an effect on cellular respiration itself. Copper seems to be capable of entering cells and, once inside, could be responsible for disrupting the biochemical processes of energy production which in insects are basically the same as in other animals, i.e. the citric acid cycle is the main pathway for the liberation of the hydrogen ion which is needed for the cytochrome system to function. And it seems reasonable that here is a likely site for copper to exert an influence since cytochrome is a copper-containing complex where excessive amounts of copper ions may upset functional equilibria.

Another aspect of this study on respiration which requires some comment concerns the effects of copper on the oxygen consumption of the three species of nymph used. It is of interest that while the three produced similar results on a general level, there are nevertheless differences. In the case of E.venosus there seems to be an all-or-nothing response in which, after a certain concentration (somewhere in the region of 20mg/l copper sulphate) is reached, the rate of oxygen consumption becomes depressed, but thereafter there seems to be little relationship between the extent of this depression and concentration. Thus, a concentration of 20mg/l copper sulphate appears to produce a greater depression in oxygen consumption than 50mg/l copper sulphate. How much this is due to experimental error it is not possible to say with any degree of certainty without more extensive experimentation with this species. What is generally accepted, however, is that there must be endless variation in the physiology of different species. These differences could be related to differences in habit which bring about different levels of activity and hence differing metabolic rates. This could certainly account for the differences in the responses of the three species studied here.

At this stage it is obvious that more research into this part of the problem needs to be undertaken to investigate further the ideas set out above. The ways in which such work could be undertaken will be discussed in a later chapter dealing specifically with future work on questions arising from this present study.

SUMMARY

1. It has been demonstrated that in the species of mayfly nymph E.venosus, B.rhodani and R.semicolorata the respiration rate as measured by oxygen consumption is depressed by exposure to certain copper solutions.

2. The effect begins at about 20mg/l copper sulphate,although in the case of R.semicolorata a slight depression occurs in a concentration of 10mg/l copper sulphate.

3. For the species B.rhodani and R.semicolorata there seems to be a graded response in that more copper causes a more acute depression. No evidence of this being the case for E.venosus was found, where only a very slight effect was caused by the copper.

4. Various ideas are discussed concerning the possible implications of these findings. These are:
   a) The effect is probably due to the respiratory system itself being affected.
   b) This effect on respiration could be indirect in that the primary effect is on nervous tissue which is responsible for the control on aspects of external respiration.
   c) The effect could be a direct one acting on either the haemolymph or on cellular respiration itself.
   d) A combination of all these factors could be responsible for the effect.

5. The results underline the differences which exist in the physiology of even closely related species.

Chapter 8

RATE OF UPTAKE OF COPPER

INTRODUCTION

In Chapter 6 it was demonstrated that copper enters the nymphs in quite large quantities and is then distributed to various tissues within the body. No attempt was made here to show the rate at which copper is being taken up. The evidence of Chapter 6 was, therefore, essentially a static picture of the uptake of copper. The work reported in this chapter is an attempt to gain some evidence regarding this rate of uptake. The desirability of such information can be summed up as follows:

1. Such information would give a more precise indication of the time scale involved in the bringing about of mortalities in a population of nymphs if the uptake rates could be correlated with data obtained from toxicity tests.

2. A dynamic picture of copper uptake generally could be of use as a basis for the formulation of guidelines concerning the applications of copper-containing preparations or wastes to natural river systems. Here, critical times where copper uptake reaches a maximum could be pointed out.

3. A knowledge of uptake rates would shed more light on the question of active excretion of copper, since it would show any equilibria reached and maintained between internal and external copper.

4. A knowledge of uptake rates could result in interesting correlations with the other parameters investigated in this study, i.e. survival rates, sub-lethal effects and effects of copper on respiration rates.

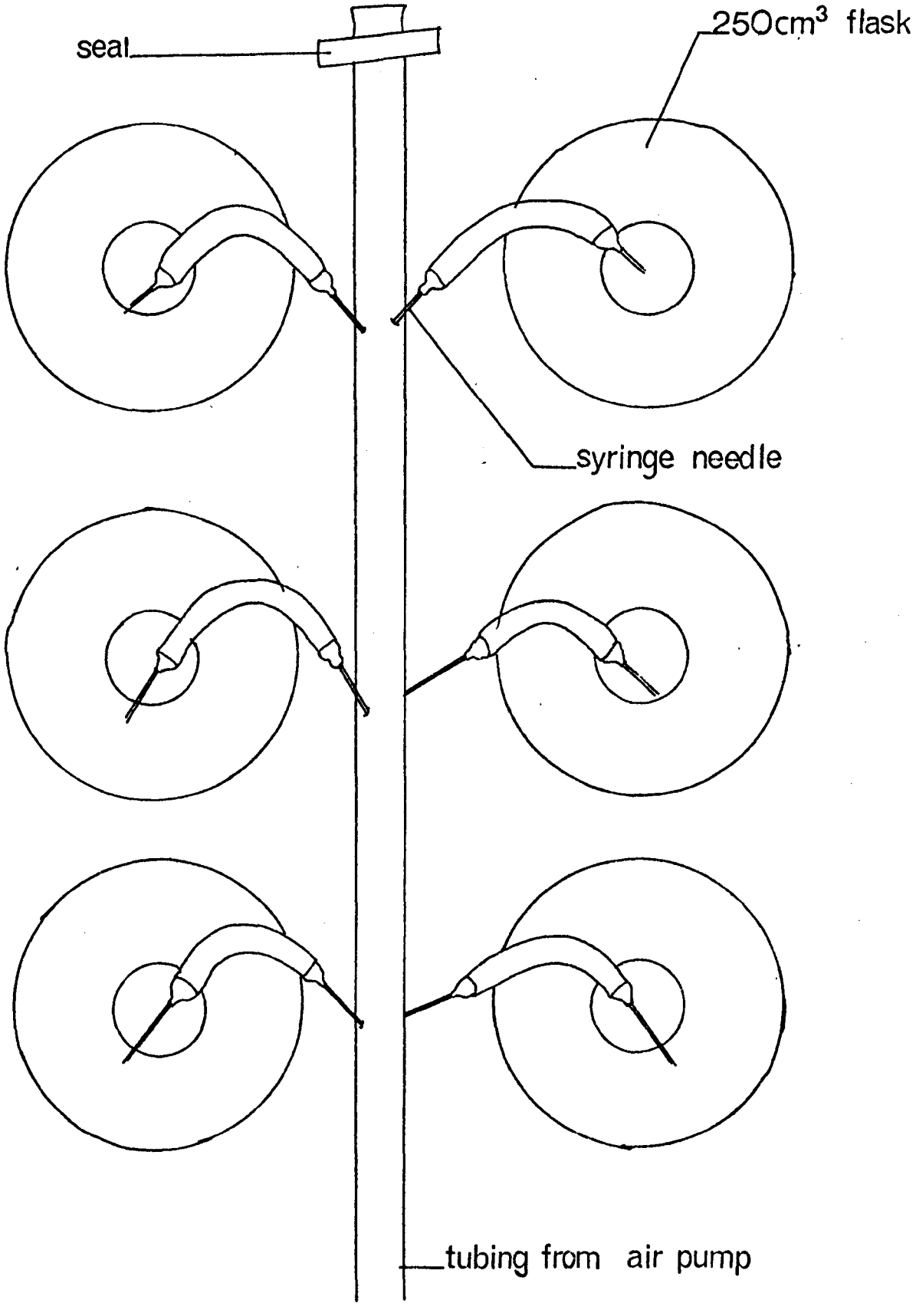
MATERIALS

It was decided that the most straightforward way of measuring the rate of copper uptake was to use the radioactive isotope Cu64. This was obtained from the Radiochemical Centre at Amersham, Bucks. and was supplied as a solution of copper chloride. This was obtained in batches of one millicurie (= 10^{-3}Curies) activity contained in $2cm^3$ copper chloride solution. Cu64 has a half life of only 12.84 hours and this was one limitation on the length of the experiments.

Nymphs of all three species obtained from the same site as that from which nymphs for the preceding parts of this study were obtained were used in this series of experiments. During the course of these experiments the nymphs were kept in 8 $250cm^3$ round-bottomed, narrow-necked 'Quickfit' flasks. This raised immediate problems concerning the aeration of the nymphs in still water, so in order to overcome these problems each flask was supplied with an aerator which was a branch of an air manifold made up of rubber tubing and hypodermic needles (Fig 32). This arrangement was found to be adequate in terms of maintaining a level of dissolved oxygen in each flask which was comparable to that used in the toxicity tests. These oxygen levels were determined by means of the Winkler method carried out during a series of dummy runs prior to the actual experiments for this reason.

The experimental flasks were submerged up to their necks in a constant temperature water bath during the course of the experiments. The experimental temperature was $18^{\circ}C \pm 2^{\circ}C$. It was not possible to

FIG 32 RATE OF UPTAKE APPARATUS



carry out the experiments at a lower and more desirable temperature since the necessary cooling unit was not available. The experiments were therefore carried out at a higher temperature than ideally desirable and one which was subject to some fluctuation according to ambient temperatures.

Each of the flasks was marked with the time interval at which samples of solution were removed from that flask as follows: 0,2,4, 6,8,12,16,24 hours. The solutions contained in these flasks were of copper chloride, since this was the salt of copper in which the Cu64 was supplied. The concentrations used were such that the copper content corresponded to that used in previous tests so that comparisons could be made. The concentrations used were 2.7 and 17.8mg/L Cu . To each of these flasks was added radioactive copper so that its disappearance from the solutions could be measured against time. To each flask, therefore, was added $0.2cm^2$ of the radioactive copper chloride using a pipette fitted with a safety bulb. A curie as defined by the I.C.R.U. is 3.7×10^{10} nuclear transformations per second. The activity of the Cu64 used here was therefore initially: 3.7×10^7 (i.e. 1 millicurie). Therefore, if $1cm^3$ of copper chloride solution contained Cu64 of activity 3.7×10^7 (or 37,000,000 nuclear transformations per second), then $0.2cm^3$ copper chloride will contain Cu64 of activity equal to 7,400,000 nuclear transformations per second; i.e. theoretically each experimental flask was receiving Cu64 of this activity at the start of each experiment. This is a theoretical value, since the Cu64 would have already undergone some degree of decay before its arrival

at the laboratory. Assuming, however, full activity at the start of each experiment, after twelve hours the activity would have dropped to 3,700,000 and after a further period of twelve hours to 1,850,000 nuclear transformations per second. Theoretically, therefore, it should have been possible to extend the experiments over a period of 24 hours. In practice, however, it was found that activity levels dropped to too low a level after about sixteen hours, and depending on the time taken for the Cu64 to arrive at the laboratory this time was sometimes even shorter. Also it was found that the nymphs began to die at or soon after 16 hours. This could have been due to a combination of several possible factors:

1. Lack of oxygen due to the aerators not being a suitable means of aerating the water. This is unlikely since, as mentioned before, the oxygen levels of the water were high enough to satisfy the oxygen demands of the nymphs, but there could have been a slight effect due to lack of mechanical stimulation to the gills.
2. The relatively high temperatures at which the experiments were carried out. WHITNEY (1939) found that Mayfly nymphs from running waters were less resistant to high temperatures than those from still waters.
3. The radioactive copper itself being the cause of death. It is possible that the Cu64 is exerting a detrimental effect on the nymphs.

The instrument used for counting was an 'Ekco' automatic scintillation counter. Using radioactive samples of known activity

this instrument was found to have an efficiency of about 70% and this set a further limit on the duration of the experiments, since at 24 hours the counter would, theoretically, be registering only 1,295,000 nuclear transformations per second of the total activity of the Cu64 at the start of an experiment.

PROCEDURE

Each 250cm^3 flask had ten nymphs of as uniform size as possible placed into it at the start of each experiment. An additional flask containing isotope but no animals was also included in each experiment to act as a control and to give some measure of the natural decay rate of the isotope during the time span of the experiment. The aeration apparatus was switched on about six hours before the isotope was added to the solutions in the flasks. The nymphs were added immediately after addition of the isotope to the flasks. During the whole course of the experiment the experimental flasks were kept partially submerged in the water bath, although it was sometimes necessary to remove individual flasks to check for mortalities. Ordinarily, however, it was possible to remove water samples and animals from the flasks without disturbing them.

At the time intervals 0,2,4,6,8,12,16 (and in one case 24) hours, 5cm^3 of solution was removed from the flasks,using a safety pipette, and transferred to a glass counting tube. This was sealed with a plastic cap and placed in the counting well of the scintillation counter. The activity was measured over a 100-second period and noted. The nymphs were then removed from the flasks, washed in distilled water and dried on a filter paper in a drying oven. The drying time was for ten minutes at a temperature of 85°C and was intended to remove surface water from the bodies of the nymphs; in the subsequent calculations, therefore, uptake was worked out in terms of wet weight. After surface drying the nymphs were macerated in a solid watch glass using a glass rod and a little clean

sand. The resulting homogenate was diluted with distilled water to make up a volume of 40cm^3. This was then stirred vigorously for 3 to 4 minutes and after allowing solids to settle out, 5cm^3 samples were removed using a clean safety pipette. These were placed in counting tubes and the activity measured. Lastly, samples from the control flask were taken and measured. At any one time interval this procedure was carried out with six samples from each of the three components of the experiment, i.e. the nymphs, the bathing solution and the control flask. The results obtained from these experiments therefore represent the means of six measurements at each time interval with their standard error. Counts of background radiation were made throughout this series of experiments.

When all samples were measured at each time interval all the pipettes and beakers used were thoroughly washed several times with dilute detergent solution and rinsed several times with distilled water and then dried in a drying oven. Other pieces of glass apparatus were similarly treated except for the counting tubes. In this case a fresh tube was used for each sample at each time interval.

The copper chloride solutions used for these experiments were made up to concentrations of 3 and 20mg/l copper chloride to give uptake rates which would be representative of the situation at high (corresponding to Series A), and low (corresponding to Series AA), concentrations. To make comparisons possible equivalent values for copper sulphate and copper chloride are given below:

3mg/l copper chloride = 4.1mg/l copper sulphate = 2.7mg copper/litre

20mg/l copper chloride = 27.0mg/l copper sulphate = 17.8mg copper/litre.

Mortalities during the experiment

It was necessary to keep a careful check of mortalities during the course of these experiments. Dead nymphs were removed and a note made of the time. They were immediately macerated as described above (after drying) and this data was used in subsequent calculations. Mortalities up to twelve hours were very rare but their incidence increased towards the sixteen hour interval, this being especially true in the case of the experiments carried out at 20mg/l copper chloride. However, in no case did the mortality of test animals exceed 50% of nymphs being used. In several of the experiments a flask containing copper chloride solution but no isotope was included and this produced a decrease in the mortality of nymphs (in 20mg/l copper chloride an average of 3% mortality resulted). It seems likely, therefore, that the isotope itself is exerting some kind of detrimental effect on the nymphs, although the evidence mentioned here cannot be regarded as conclusive.

RESULTS AND DISCUSSION

The data obtained from these experiments were dealt with in the following ways:

1. The background counts made during the course of the experiments were used to correct the counts in respect of this factor. In most cases the background radiation was very small and probably would not have affected the results significantly.

1. The data was converted to microcuries (mc) Cu64 per gram wet weight of nymph and it is in this form that the results are expressed in the graphs that follow where mc/g. wet wt. is plotted against time. The points used for the plots represent the mean of six counts for which the standard error was calculated. A full table of results is given in the appendix (Table 18 and 19)

3. Before using the data to draw the graphs a correction factor had to be introduced. This was necessary because the natural rate of decay of the isotope had to be taken into account to give a true picture of the uptake. To correct for this the following equation was used:

$$\log_{10} No = \log_{10} Nt + \frac{0.3010\ t}{t\frac{1}{2}}$$

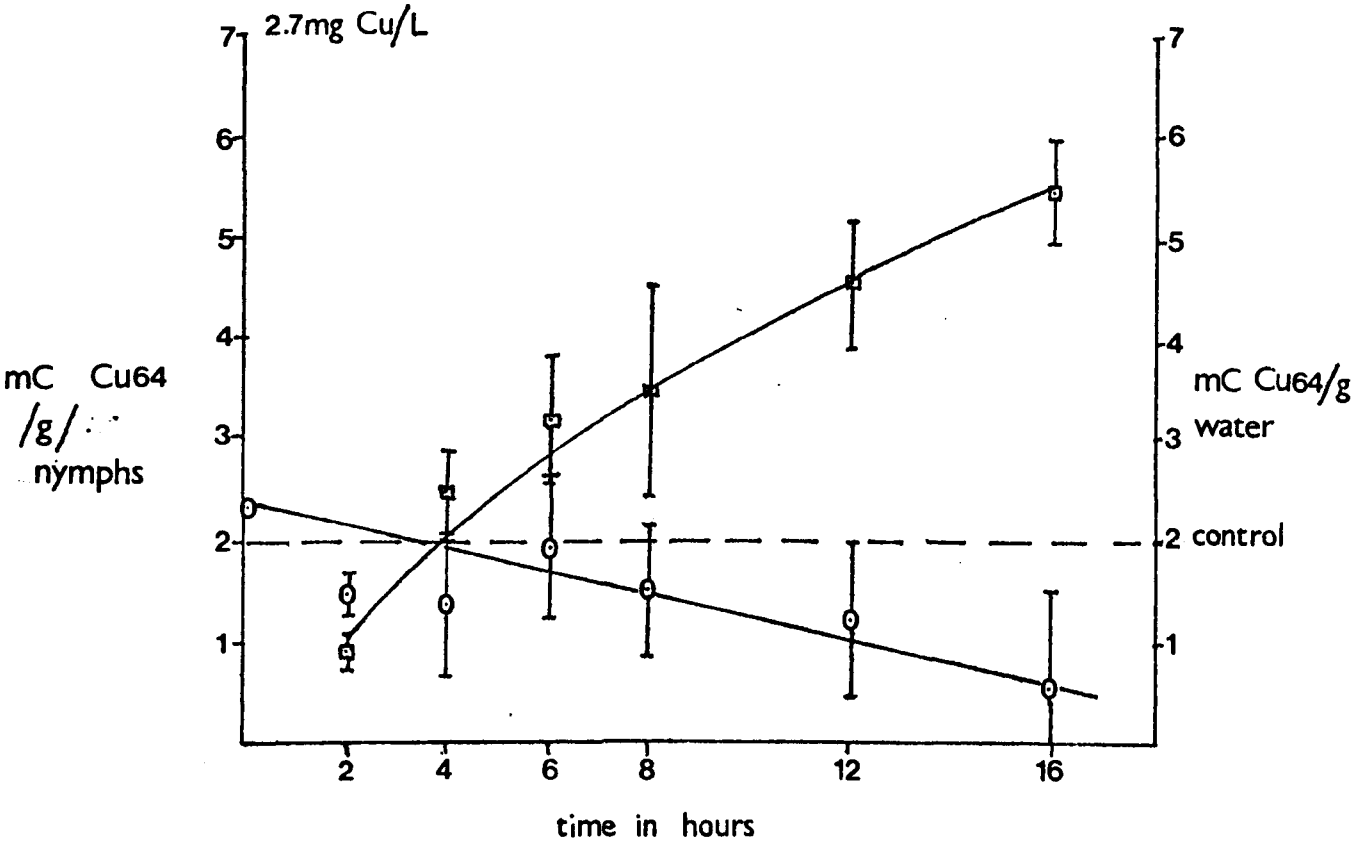
where: Nt = observed count
No = desired (corrected) count
t = time between reference point(time 0) and time of counting
$t\frac{1}{2}$ = half-life of isotope

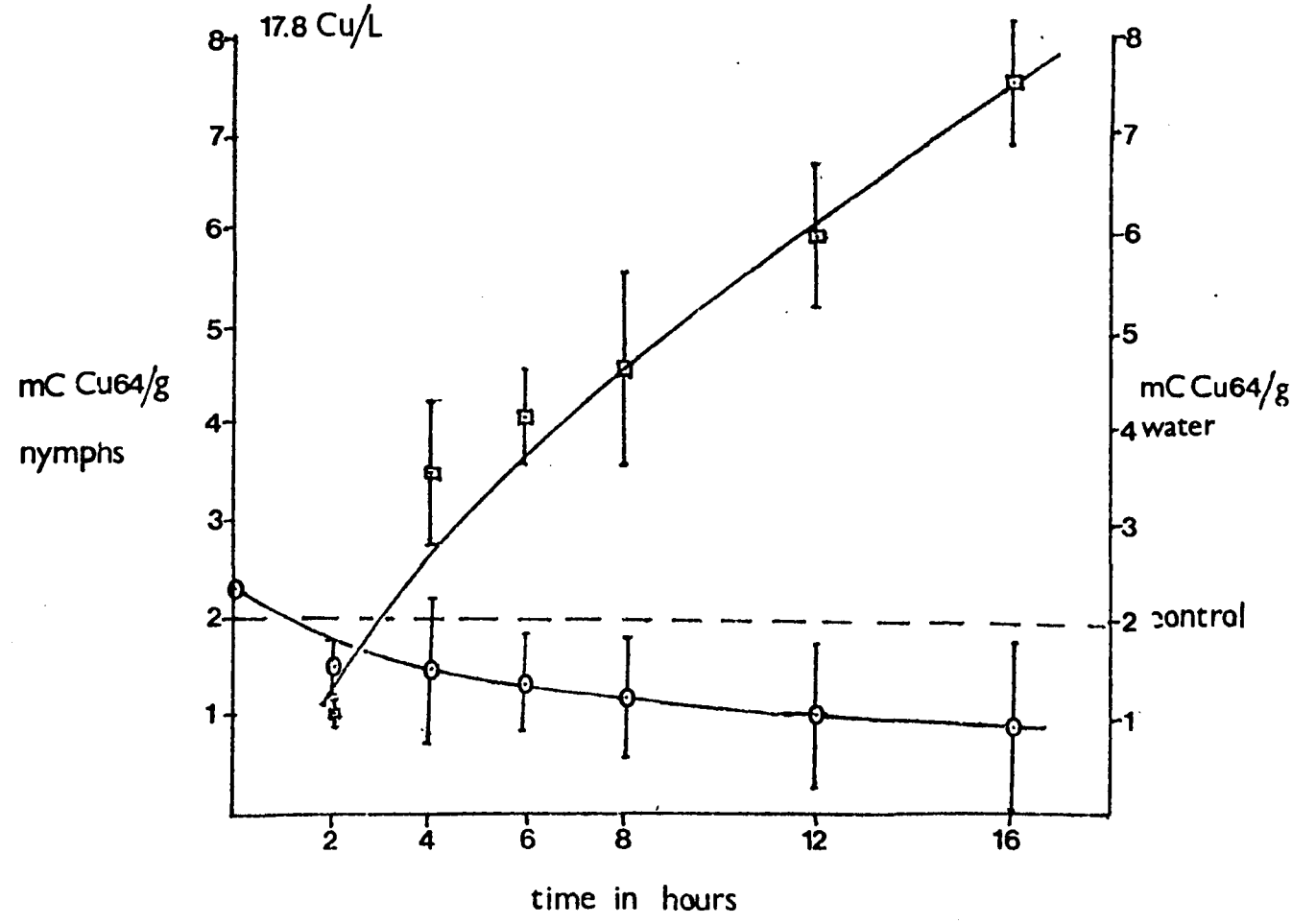
4. Using the corrected data, graphs were drawn showing the uptake for the three species at the two concentrations used. These are shown in Fig. 33 to 37 .

*

In the cases of all three species of nymph used copper is seen to be accumulated rapidly up to sixteen hours and this accumulation is greater in the more concentrated of the two copper chloride solutions used. This is interesting because it seems to suggest that uptake is due to an active process since, if it was caused solely by a passive entry of copper, one would not expect a rise in the count of radioactive copper in relation to a rise in concentration of copper chloride solution. This is because the concentration of Cu64 was the same in both solutions and so, although there might be a higher intake of non-radioactive copper since more ions are present in a more concentrated solution, this does not hold true in the case of the radioactive copper if all the copper was entering passively. An active process, however, that was for some reason accelerated would result in a greater intake of both normal and radioactive copper. Assuming, therefore, that there is active uptake of copper as opposed to, or at least in addition to, a passive, mass-flow mechanism, it is interesting to speculate how and where this is operating. The most simple explanation would seem to be that the mechanism involved is not one specific to copper or to any other ion in particular, but is brought about simply by the nymphs taking water in through the mouth, i.e. active drinking. If this process was for some reason accelerated, then one would expect an increased uptake of a wide variety of substances and ions present in the water, including radioactive ones. It has already been shown in Chapter 6 that large amounts of copper are found in the gut and

FIG 33 ACCUMULATION OF COPPER B. rhodani





nymphs ; water samples

FIG 34 ACCUMULATION OF COPPER E.venosus

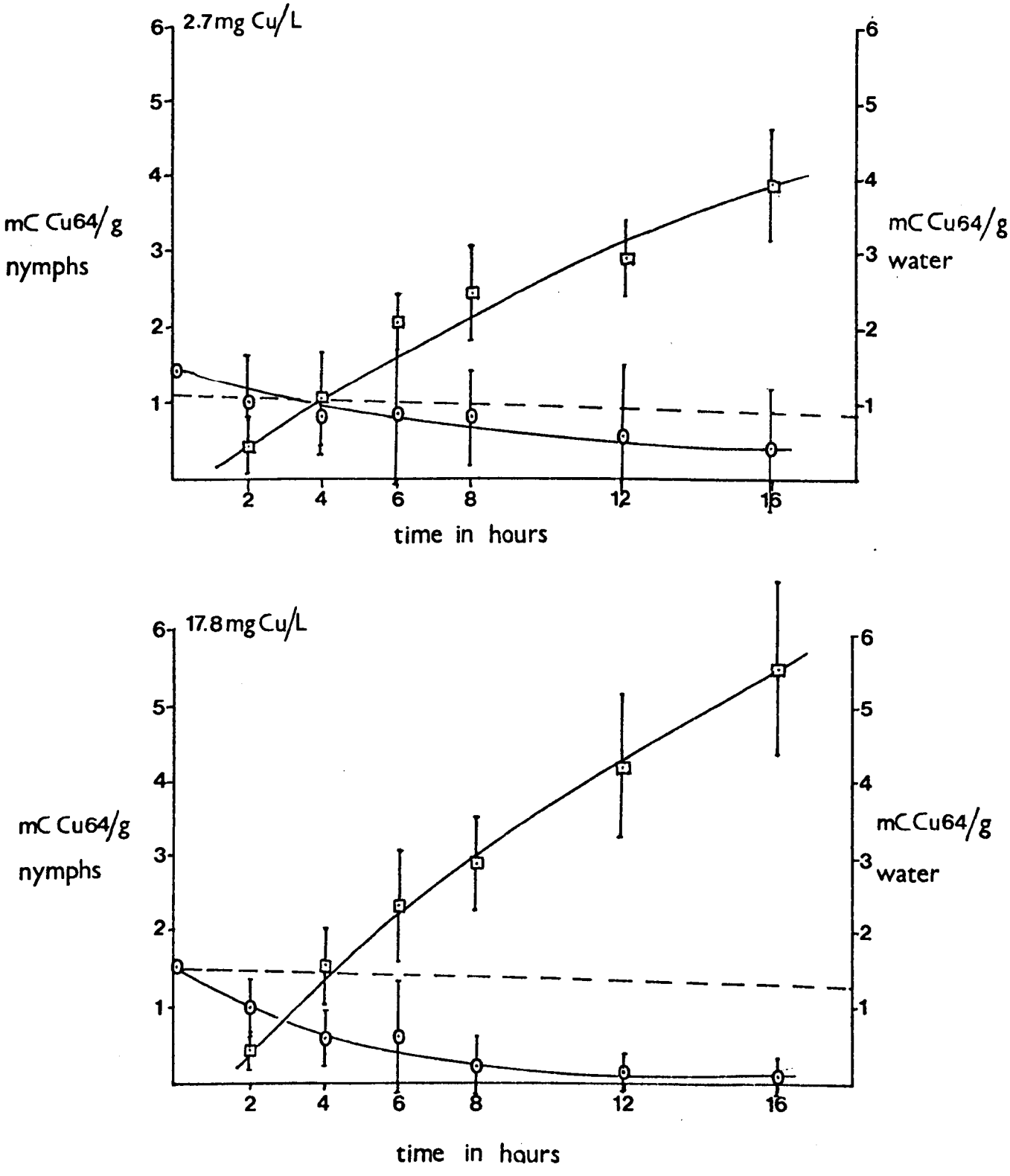
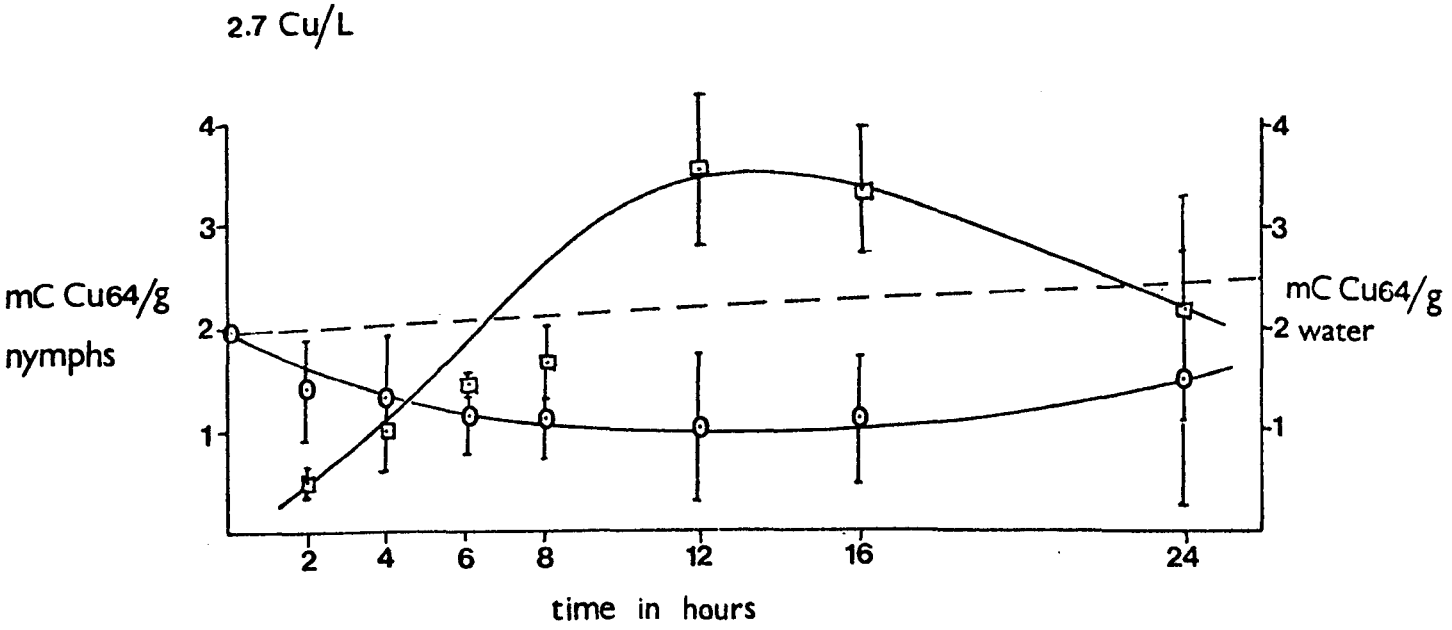
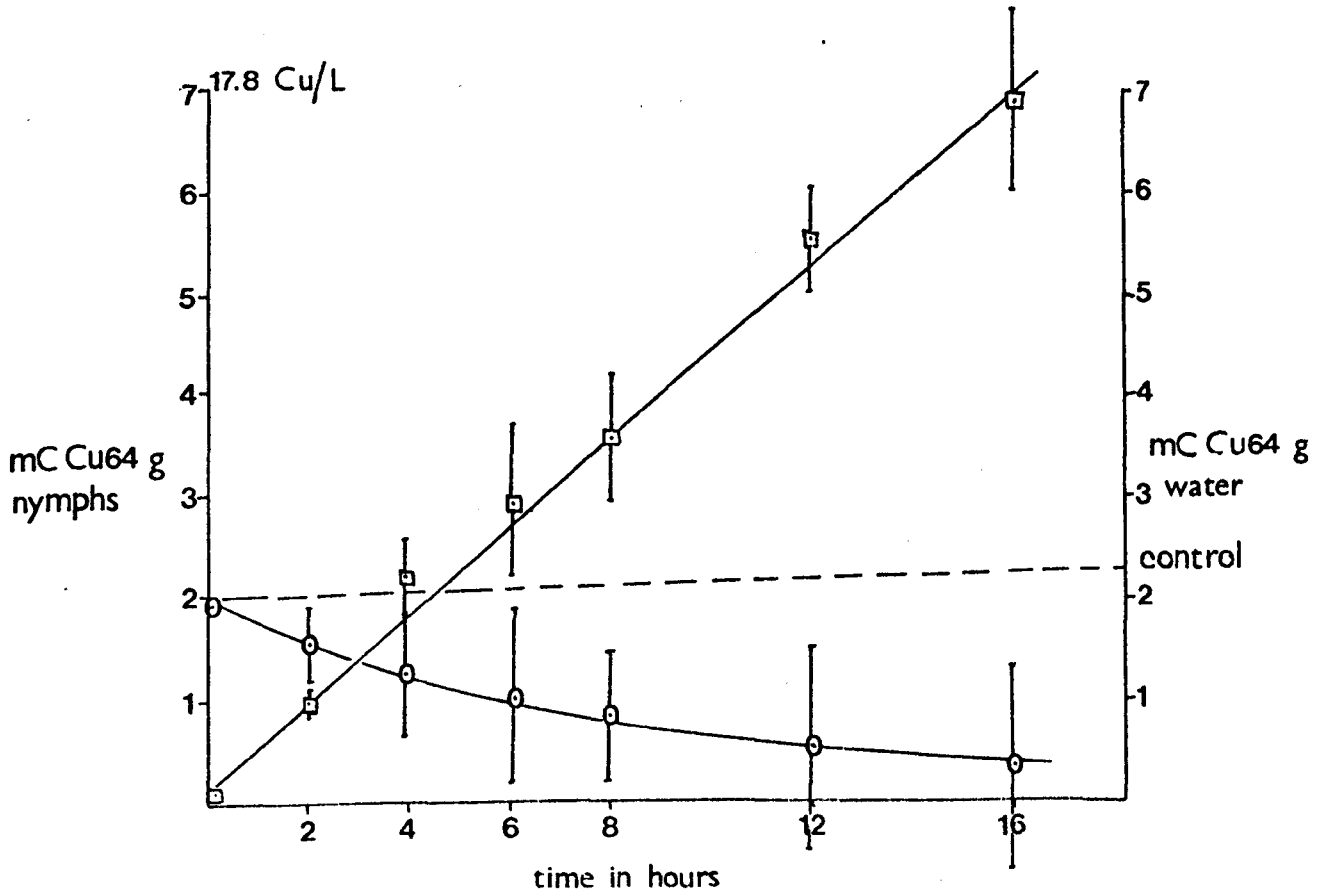


FIG 35 ACCUMULATION OF COPPER R.semicolorata





there could be no other way of entry other than through the mouth. It is suggested, therefore, that higher concentrations of copper have the effect of increasing the rate of drinking. It is not possible here to give a satisfactory explanation as to why this occurs except tentatively to suggest as one possible explanation that an increased drinking rate is only part of an initial increase in general activity brought about by copper solutions. (It might be suggested that the effect is caused by some weakening of the mouth parts brought about by copper so that the nymphs cannot close their mouths effectively. This alone, however, would not necessarily cause a greater influx of water, since some muscular contraction of the pharynx is necessary to cause water to enter the oesophagus.)

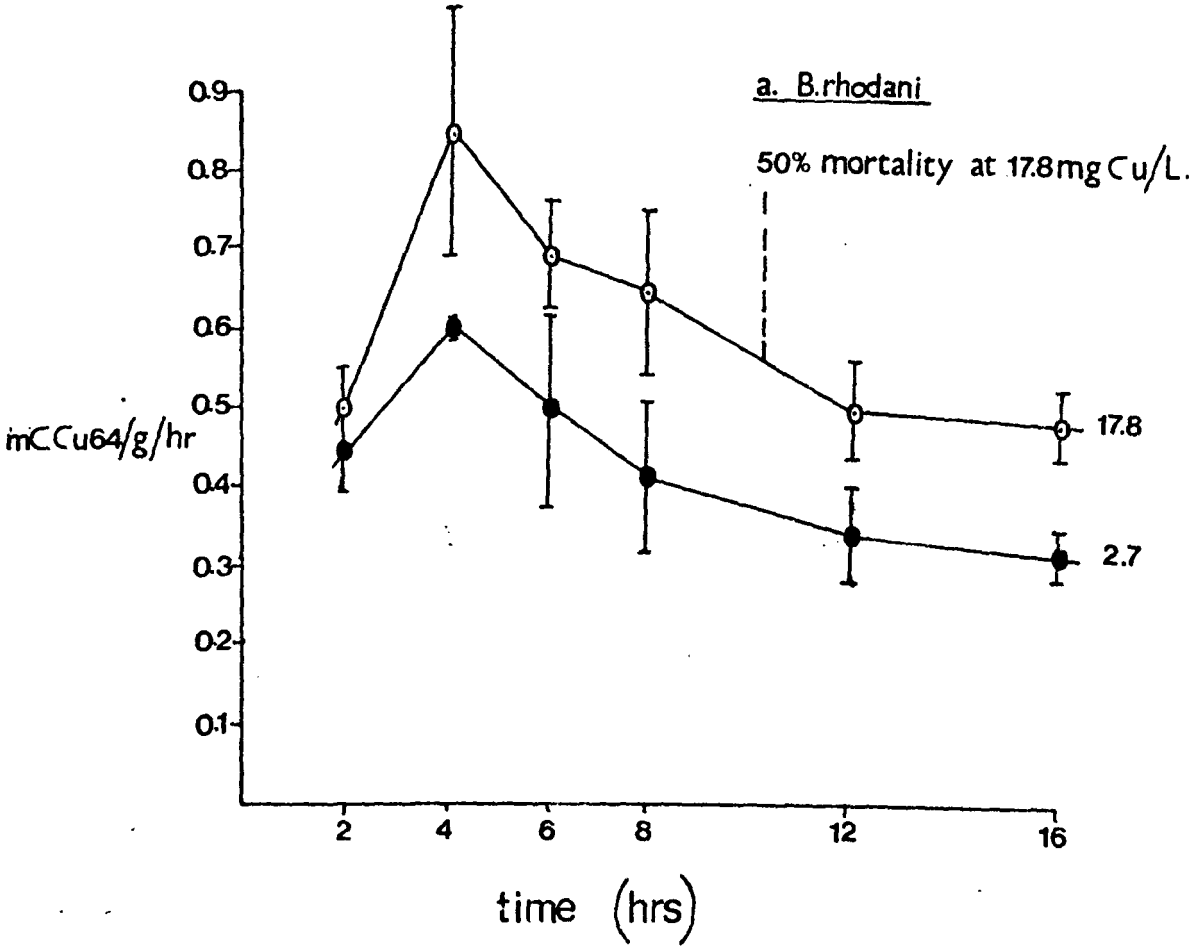
In nearly all cases the accumulation of copper is seen to be linear up to sixteen hours and this suggests that excretion of copper may either not be taking place at all or, if it is, at a very low level. The result obtained for R.semicolorata at 2.7mg/l copper (Fig.35) is difficult to explain since it is rather atypical. This experiment was carried out over a period of 24 hours and was one of the first to be carried out in this series. As can be seen, the amount of Cu64 being taken up has apparently dropped after sixteen hours. Also the general level of copper uptake is lower than for either of the other two species at this concentration. Although, therefore, these results are included it is suggested that they are somewhat unreliable since 90% of the nymphs had died in the 24-hour flask, also mortality was unusually high in the other flasks and surviving nymphs became very inactive after twelve hours in this

particular experiment. Ideally, this experiment should have been repeated but time did not allow for this.

From the data obtained, the actual rate of uptake per hour was calculated and the graphs obtained from these data are shown in Fig. 36 to 37 (the full data tables will be found in Tables 18&19 in the appendix). As can be seen, a peak of uptake occurs at 4 hours in the cases of B.rhodani and R.semicolorata (17.8mg/l Cu) and at 6 hours in the case of E.venosus (at 2.7mg/l Cu). These rates, together with the cumulative amounts of Cu64 taken up per gram, are interesting when one makes comparisons between the three species. In both cases it can be seen that B.rhodani has a higher rate of uptake than either of the other species, and at 17.8mg/l Cu E.venosus has the lowest. These results correlate fairly well with the information yielded by the toxicity tests, where it was found that B.rhodani was less tolerant to copper than either of the other two species, and it would seem reasonable to suggest that a higher initial rate of uptake at least contributes to this.

Graphs 36 to 37 also show that after the peaks of uptake have been reached they are followed by a slow decline in uptake rate, so that it seems that much of the copper which is taken up during any given period of time is taken up during a phase near the beginning of that period, although up to sixteen hours at least copper is still being taken up, but at a reduced rate. This is especially true in the case of B.rhodani (Fig.36), where there is a particularly high rate of uptake at four hours followed by a relatively rapid decline of this rate. In the cases of the other two species, although there are peaks at four or six hours these are not as pronounced and they

FIG 36 RATE OF UPTAKE



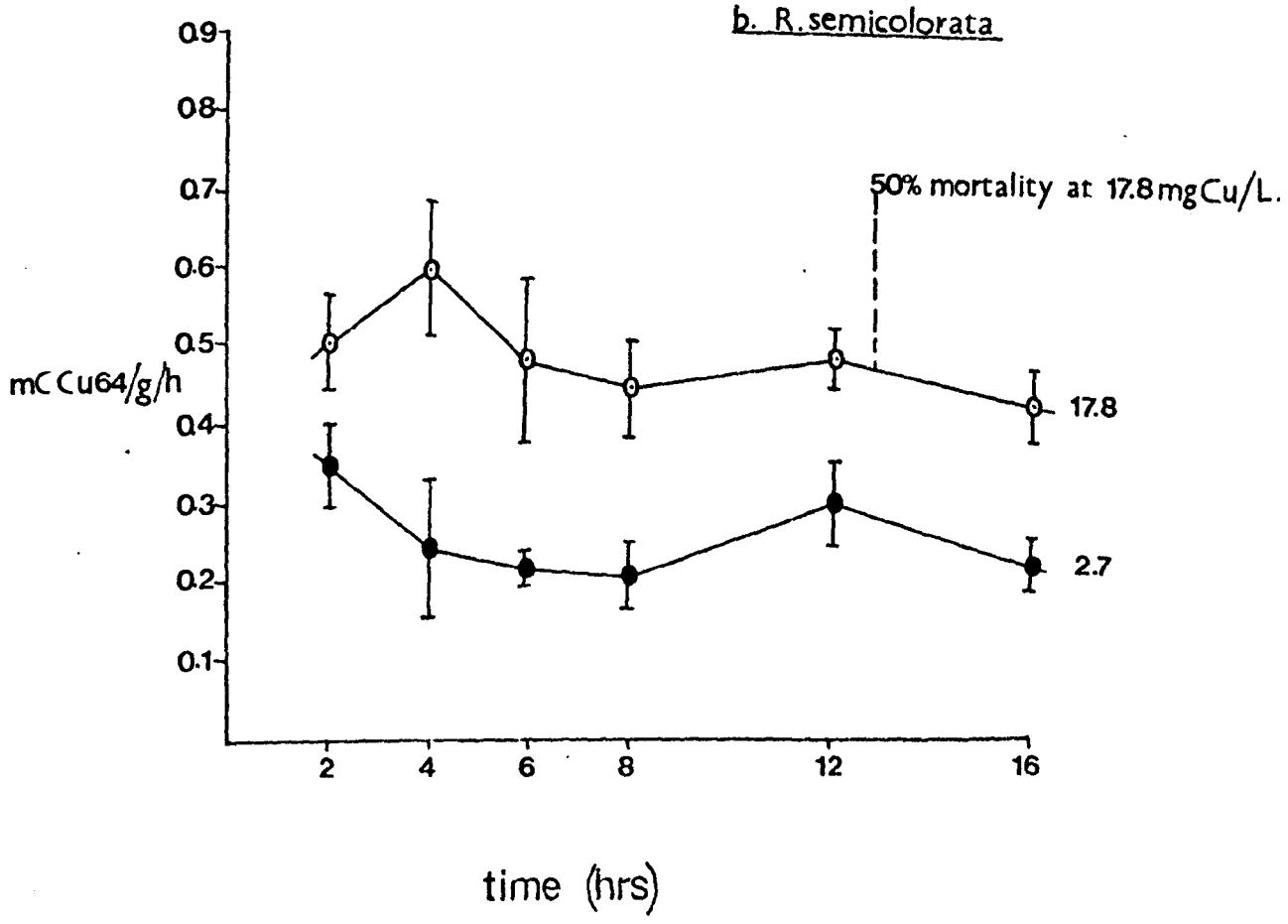
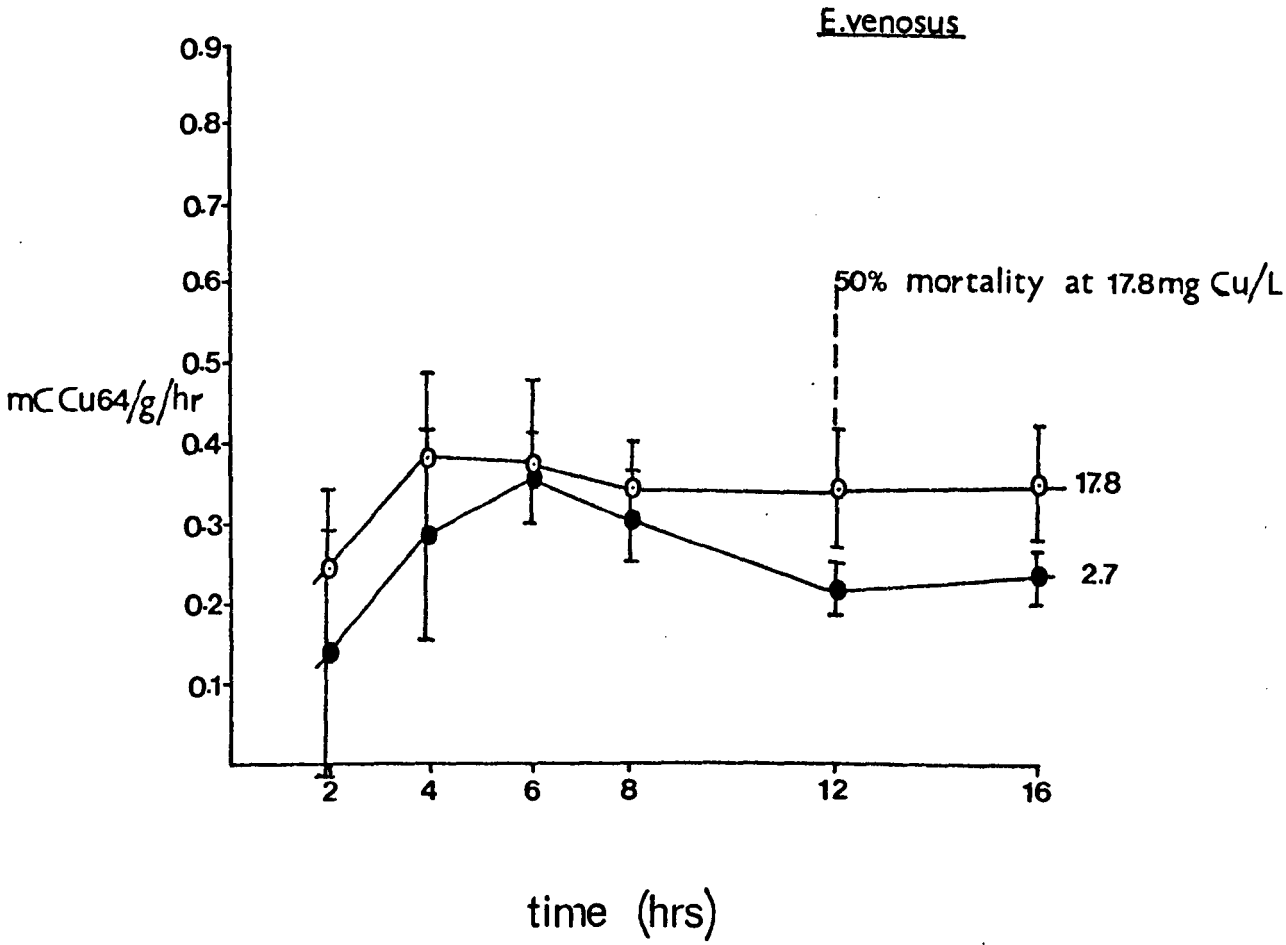


FIG37 RATE OF UPTAKE



are followed by a more steady uptake rate up to sixteen hours. Presumably a larger initial uptake will bring about a more rapid toxic effect, causing gradually reducing activity ending ultimately in death.

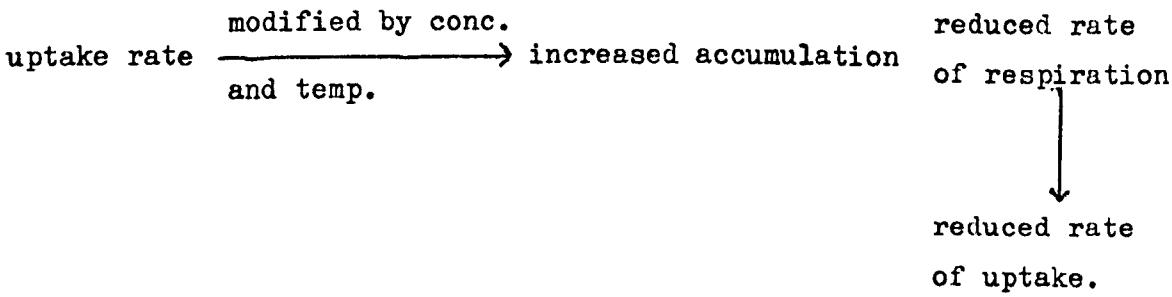
The fact that concentration affects the rate of uptake also correlates well with the toxicity test data which show that there is a direct relationship between concentration and toxicity. It is reasonable, therefore, to envisage a situation where higher concentrations will cause larger and, possibly, earlier peaks of uptake to occur. This will affect the overall toxicity because, as Fig. 36 to 37 show, 50% mortality seems to occur several hours after the peak of uptake has been reached, so that very early peaks which may result from high concentrations will be followed by a corresponding 50% mortality in a population, i.e. the higher and earlier the peak the more rapidly will a 50% mortality be reached.

The time at which the maximum rate of uptake occurs is of importance also in terms of the ability of animals to recover after short exposures to toxic copper solutions. In the case of B.rhodani, for example, a peak uptake rate is reached in four to six hours after exposure to a copper concentration of 17.8mg/l Cu. The times at which these peaks occur help to explain why short exposures of less than four hours produce much prolonged survival times for given concentrations of copper. In Series F (Chapters 4 and 5) of the toxicity tests it was found that an exposure of 2 hours to 20mg/l copper sulphate (= 13.2mg/l copper) resulted in a 50% mortality in about 220 hours, whereas an exposure time of 8 hours

produced a 50% kill in only about 24 hours (See Fig.24 , Chapter 5). It seems, therefore, that copper uptake which takes place during the relatively early stages of a time period have a far-reaching effect on the overall toxic effect that is exerted. In this case this critical time seems to be in the region of 4-6 hours for the species of nymph used in this investigation for a concentration of about 20mg/l copper. At higher concentrations this time will, presumably, be arrived at after a correspondingly shorter period of time, although more work in measuring the uptake rates of copper at higher concentrations will have to be carried out to confirm this point.

In the case of respiration rates it was shown in Chapter 7 that copper begins to depress this after about 150 minutes (about $2\frac{1}{2}$ hours) in the cases of B.rhodani and R.semicolorata (in the case of E.venosus the picture is not so clear). Here, the lowest concentration giving clear results was about 50mg/l copper sulphate (= 33mg/l copper); therefore, it is not possible to make precise comparisons since the highest concentration used in this present series of experiments was 17.8mg/l copper. However, in the case of respiration it seems more likely that the actual accumulation of copper will be of more direct relevance than the rate of uptake, although this is indirectly concerned since this causes the ultimate accumulation level. Generally, therefore, it can be said that there exists an inverse relationship between copper accumulation and rate of respiration. It is possible, also, that the decline in the rate of uptake of copper which takes place after about 4-6 hours is in turn caused by the declining rate of respiration, assuming that this

is accompanied by a reduced rate of activity. These three parameters, accumulation, rate of uptake and rate of respiration are, therefore, seen to be closely interrelated and, further, these relationships are affected by external variables such as copper concentration and (probably) temperature. The relationships can be summed up as follows:



SUMMARY

1. The uptake of copper by the three species R.venosus, B.rhodani and R.semicolorata has been studied using the radioactive isotope of copper Cu64.

2. Copper has been shown to be accumulated rapidly within a period of sixteen hours. The accumulation is such that it shows no evidence of copper excretion taking place to any great extent. This is not, however, conclusive, especially in the light of the fact that copper was found to be present in the Malpighian tubules of the nymphs (Chapter 6).

3. For all three species the rate of uptake follows a similar pattern in that a peak of uptake is reached at about 4-6 hours and thereafter the rate begins to decline. This peak was the most pronounced for B.rhodani.

4. It has been shown that concentration of external copper has an effect on the rate of uptake in that in more concentrated solutions more radioactive copper is taken up by the nymphs. It is suggested that this is evidence for there being an active mechanism involved in the uptake process.

5. It has been shown that the three species yield slightly differing results which correspond reasonably well with the pattern of specific reactions to copper found throughout this study.

6. The rate of uptake has been compared with the information derived from the toxicity tests and a relationship between the peaks of uptake and mortality has been found to exist.

7. An explanation is offered for the results obtained from Series F (Chapter 5) in terms of peaks of copper uptake.

8. An interrelationship between accumulation, uptake rate and respiration rate is described.

Chapter 9

CONCLUSIONS AND SUMMARY

CONCLUSIONS

THE EFFECT

In his study of the distribution, abundance and life histories of Stoneflies and Mayflies, LANGFORD (1971) stressed the fact that these two groups are generally regarded as the most sensitive to ecological disturbance. This study has shown that three species of Mayfly nymph, although in relative terms showing a certain degree of tolerance, are sensitive to copper, the introduction of which into natural waters must be regarded as constituting an 'ecological disturbance'. The nature of this sensitivity is in terms of chronic toxicity, and threshold concentrations of toxicity have been found for each species. These are in the order of 1.0mg/l copper and it is easy to imagine a situation in which the amount of dissolved copper in a natural freshwater system will exceed this value. The maximum limit for copper set by the USPHS in 1962 was 1.0mg/l, but this was only a recommendation and applied to drinking water only. The first conclusion, therefore, is that the three species of nymphs studied here and, by implication, all species of mayfly nymph to a greater or lesser extent, are susceptible to what in polluted natural conditions must be regarded as fairly small amounts of copper. The thresholds of toxicity found in this study must often be surpassed in waters receiving effluents containing copper, so that populations of mayfly will be detrimentally affected. Also these thresholds will be affected by temperature so that at higher temperatures the threshold concentrations will be lowered, and added to this, other complicating factors such as pH and water hardness will affect the threshold

values. It must be generally concluded, therefore, that populations of Mayfly nymph will be at risk through the uncontrolled discharging of copper effluents into natural freshwater systems.

Depending on the concentration of copper, rapid uptake takes place up to six hours, after which recovery is not likely. Therefore, even relatively short exposures to abnormally high concentrations of copper will cause a high mortality rate in that population. It is possible, however, that this effect will be reduced because cupric ions introduced into natural waters at pH 7 and above will quickly precipitate as the hydroxide or as basic copper carbonate, $CuCO_3.Cu(OH)_2.H_2O$. In this case, therefore, copper will be removed by adsorption and/or precipitation (McKEE and WOLF, 1963). In a river which is continually receiving algicidal preparations containing copper, or one which is receiving a continuous discharge of copper-containing effluent, there will always be an appreciable amount of copper in solution at any one time, so that precipitation may not be rapid enough to reduce its effects appreciably.

A general conclusion that can be drawn from a consideration of several factors is that it is difficult to predict exactly what the toxicity of copper will be when introduced into a river or stream without a very careful study of the physical makeup of the water into which the copper is being introduced. This is because the effect which a given concentration of copper will have on a population of Mayfly is subject to several physical factors and the net effect will depend on the interaction and superimposition of these factors.

Added to this must be the question of sub-lethal effects. So far only chronic toxicity has been discussed but it is possible that certain sub-lethal effects which are not immediately noticeable will also occur, and until these are fully investigated it is only possible to formulate a very incomplete prediction of the effects of copper.

Copper affects the rate of respiration by depressing it and this effect is subject to interspecific variation. Respiration itself can be regarded as a sub-lethal factor since a reduced respiratory rate that does not necessarily lead to death within a short space of time will nevertheless affect other aspects of the nymph's normal life pattern. One factor which will almost certainly be affected by a reduction in respiration is activity. In the natural environment a reduction in general activity will have far-reaching effects in terms of escape from predators, maintenance of position in the stream and the finding of food. Correct orientation with respect to light and current may also be affected by a reduction in activity.

In considering the different factors which have a modifying effect on the basic copper toxicity as determined by the toxicity test at the beginning of this study, a rather complex picture begins to emerge and perhaps a general conclusion for this part of the work is that the responses which are likely to occur towards a pollutant such as copper are subject to modification by a complex interrelationship of physical and biotic factors. Certainly the biotic factors in terms of sub-lethal effects are the least known and until these are better understood for this group of animals only conclusions

containing a certain amount of speculation can be drawn.

THE CAUSE

It is concluded that the cause of copper toxicity in this case springs from internal, physiological effects rather than those arising from external surface effects. This conclusion stems from two pieces of evidence. The first is the fact that copper has been seen to enter the nymphs in large amounts and is then distributed to various tissues of the body including the central nervous system. The second is related to the first and is the fact that the rate of uptake of copper is rapid, with a correspondingly rapid accumulation. Copper is therefore seen to be entering the nymphs. No evidence was found to suggest any surface deposits or accumulations of copper either on the general body surface or on the gill surfaces. Once within the internal tissues copper may, therefore, disrupt several processes. One of these is related to respiration since it has been found that copper in trace amounts inhibits the respiration of Mitochondria (PETERS and WALSHE,1962) by blocking pyruvate oxidation. The amounts of copper involved here are minute, being only 5g, so that amounts far below the threshold of chronic toxicity could exert detrimental effects on the respiration of the nymphs. Another possible site of action is concerned with membrane permeability, since it is known that heavy metals, including copper, have the power to inhibit facilitated transfer and also to increase membrane permeability to potassium ions (DAVSON,1970). This is particularly interesting when added to the fact that copper is accumulated in the

central nervous system which has as a functional basis the transfer of sodium and potassium ions across cell membranes. It seems reasonable, therefore, to suggest that copper is exerting an effect here. Also, it has been shown that copper inhibits membrane permeability to chloride ions (CHIARANDINI et al,1967), so that some general effect on membrane permeability vital to the maintenance of ionic equillibria can be expected although, until work is carried out specifically on the three species used in this study, this is only a tentative suggestion. Another implication of interference with membrane permeabilities is that animals may not be able to maintain osmotic balance with their external environment, a factor which is vital to animals living in freshwater.

In general terms the causes of toxicity can be seen to stem from several possibilities each contributing towards the overall effect. These possibilities can be summed up as follows:

1. Depression of respiration rate which could be due to inhibition at mitochondria level.
2. Disruption of membrane permeabilities generally, thus making normal osmotic regulation impossible. This would also put the excretory system out of action.
3. Disruption of membrane permeabilities specifically in nerve cells resulting in disruptions of coordination generally. This point is also related to respiration, since, as already described, respiratory movements are controlled by the segmental ganglia.

It is suggested that there is no single cause of copper toxicity but that the effect is a composite one with each component contributing towards the ultimate death of the nymphs in the case of chronic toxicity, or to longer-term detrimental effects in the case of sub-lethal poisoning.

These conclusions can be summarized as follows:

1. The three species of Mayfly nymph used in this study, although showing a certain degree of tolerance, are nevertheless sensitive to copper at certain concentrations.

2. The species show a sensitivity, in terms of acute toxicity thresholds, to concentrations of copper in the region of 1 mg/l.

3. Mayfly species in the natural environment are at risk from pollutants containing copper.

4. The degree of effect which a population exposed to copper will suffer is subject to modification by several factors including temperature, pH and water hardness.

5. Copper is taken up rapidly within a period of six hours from the start of exposure. This rate is affected by the external concentration of copper.

6. It is difficult to predict exactly the extent of copper toxicity in the natural environment as the net effect is dependent on several interacting factors. These include sub-lethal effects.

7. Copper affects the rate of respiration by depressing it for at least two of the species used.

8. The cause of copper toxicity is a physiological one as opposed to an external surface effect.

9. Several possibilities for this physiological cause are discussed and it is suggested that there is no single cause for chronic copper toxicity.

SUMMARY

1. The literature relating to copper toxicity in terms of toxicity test methodology, and treatment of data obtained from toxicity tests, has been reviewed.

2. A short review of the literature regarding the general biochemistry of copper has been included in this study.

3. The nymphs used and certain aspects of their ecology have been described.

4. Laboratory studies have been carried out on various aspects of copper toxicity to the nymphs of the Mayflies, Baetis rhodani, Ecdyonurus venosus and Rithrogena semicolorata.

5. A preliminary series of toxicity tests have been carried out using all three species to establish basic profiles of copper toxicity at different concentrations. From the data obtained from these tests Tlm survival curves were drawn. It was found that a simple relationship exists between concentration and toxicity in that more concentrated copper solutions had a more toxic effect. Also it was found that the three species did not have exactly the same tolerance to copper. B.rhodani was found to be the most susceptible, followed by E.venosus, with R.semicolorata as the most tolerant of the three.

6. A series of toxicity tests at low concentrations of copper were carried out to ascertain threshold concentrations of toxicity.

The results were used to draw Tlm curves and the following threshold of toxicity values were found:

B.rhodani	0.48mg/l Cu
E.venosus	0.68mg/l Cu
R.semicolorata	0.76mg/l Cu

The differential susceptibilities are again stressed.

7. A series of toxicity tests designed to confirm that the toxic effect is due to copper and not the sulphate radicle in the above tests was carried out. In this series other salts of copper were used, these being copper nitrate and copper chloride. The results of these tests were subjected to a probit analysis and it was found that there is no significant difference in the toxicities of the different salts. A further experiment using magnesium sulphate was carried out in which it was found that this salt had no toxic effect on the nymphs. It was concluded that the toxic effect of copper salts is indeed due to the copper.

8. A series of toxicity tests designed to determine the effects of certain complicating factors on the toxicity of copper was carried out. The factors investigated here were temperature, pH and hardness. The data obtained from these experiments were subjected to a probit analysis and the results demonstrated that the toxicity of copper is increased at higher temperatures and at low pH values. This toxicity is, however, decreased in waters of increasing hardness. A brief description of these variables in natural waters is given.

9. A series of toxicity tests to determine the effects of short exposures to copper was carried out. Here nymphs were exposed to different concentrations of copper for times ranging from 2 to 12 hours, depending on the concentration of copper. It was found that recovery is possible only with short exposures (2 hours) to low concentrations (10mg/l copper sulphate = 6.6mg/l copper). In the case of longer exposures the effect was merely to prolong the time taken for a 50% mortality. The conclusion drawn is that exposures of 4 hours and over to even low concentrations are ultimately toxic but that this toxic effect is spread out over a protracted period of time.

Only the nymphs of B.rhodani were used in this series.

10. Serial sections were made of the bodies of nymphs killed by toxic copper solutions and these were then treated with the histochemical stain, Rubeanic acid. Photomicrographs were made from these preparations which clearly show that copper is being taken into the gut of the nymphs and is being accumulated in various tissues including those of the central nervous system. Other areas of copper accumulation were found to be the malpighian tubules, the gill filaments and the fat body.

The possible implications of copper in these regions are discussed. No evidence of external surface adsorption of copper was found.

11. A short review of the methods used for measuring respiratory rates is given.

12. Experiments were carried out to show the effect of copper on the respiratory rates of the three species of Mayfly nymph used in this study. For this purpose a continuous flow respirometer linked to a highly sensitive micromanometer was used.

It was found that in the case of the species E.venosus the effect on respiration was only slight. For the other two species there was a definite depression of respiratory rates which was related to copper concentration, more concentrated copper solutions producing a greater depression in respiratory rate as measured by oxygen consumption.

The implications of these findings are discussed.

13. The radioactive isotope of copper Cu64 was used to measure the uptake rates of copper in the three species.

It was found that up to a period of 16 hours accumulation of copper is rapid but that the rate of uptake causing the accumulation reaches a peak at 4-6 hours and then gradually falls off. The rates of uptake for each of the three species compares well with their different susceptibilities to copper, B.rhodani having the most rapid rate.

These rates are compared to earlier findings and some correlations between the Tlm curves, short exposure experiments and respiration rates are discussed. It is suggested that these findings show that the uptake of copper is not passive but is at least linked to an active process.

14. The correction factors and statistical methods used in processing the data from the Cu64 experiments are described.

15. Recommendations for further study are discussed.

16. It is concluded that the effect that copper will have on a given population of Mayfly nymphs is subject to several variable factors which must be carefully considered when making any predictions regarding such effects.

It is suggested that the toxicity of copper is brought about by a complexity of factors rather than one single cause. Here copper is responsible for the disruption of several vital physiological processes.

Chapter 10

RECOMMENDATIONS FOR FURTHER STUDY

1. In general terms this study has shown that the three species of Mayfly nymph used, although closely related, are nevertheless affected to significantly differing extents by copper. This is true in the case of the direct effect of copper as expressed by survival curves or in the case of the effects of copper on a physiological process such as respiration. This observation would, therefore, seem to lead one to suppose that in an ecosystem consisting of many thousands of species of invertebrates, some of which will not be closely related, one can expect a spectrum of responses to a pollutant such as copper. At present there is very little information regarding the susceptibilities of freshwater invertebrates to heavy metals and a broad programme of studies taking in the effects of several heavy metals on a wide variety of invertebrate species would seem to be desirable. The starting point for this study could be the toxicity test, so that basic profiles of tolerance of a wide range of animals would be built up.

2. This study has been carried out using three species of Mayfly nymph which are found in fast-running waters, and it seems desirable that some work should be carried out with still water and burrowing species since this might uncover differences in response arising from different modes of life. Studies on the effects of copper on the respiration of still water species would make a particularly interesting comparison with running water species.

3. This has been a purely laboratory study and some work in the field would shed more light on the toxicity of copper in the natural environment. Bioassays could be carried out in natural waters with

known high copper contents to give information regarding the effect of this metal on species variety, distribution and number. Food webs constructed from such information would stress the implications of heavy metal contamination in freshwater populations.

4. Studies have been carried out into the effects of heavy metals, including copper, on the behaviour of fish (JONES,1947) and it seems that the techniques used here could be adapted for use with Mayfly nymphs. The behaviour studied would centre around avoiding reactions and it should be possible to set up and maintain sharply differentiated concentration gradients of copper solutions in the laboratory. In this way it would be possible to discover whether the nymphs are, firstly, sensitive to small concentrations of copper in that they are able to detect its presence, and secondly, whether they exhibit avoiding reactions which will determine their spatial distribution. Such avoiding reactions would be of some importance in the natural environment, especially if they result in migrations of nymphs to less favourable reaches of a river in terms of dissolved oxygen and food availability.

5. It has been demonstrated in this study that copper is less toxic in hard waters and this effect can be explained in terms of an antagonistic effect between the copper and calcium ions. It would be of some benefit to carry out further work on antagonism in relation to copper with a view to finding other ions and substances which would have an antagonistic effect. Many substances make up the physical aquatic environment and the effects of these on copper toxicity would be of interest.

6. A more detailed and extensive study of the actual mechanism of copper toxicity should be undertaken. In this present study it has been demonstrated that for two of the species studied, copper depresses the respiratory rate. This observation could be used as a starting point for a more thorough investigation into this aspect of the problem. It is known, for instance, that the blood of insects does have a small role to play in the transport of oxygen. Some study into the effect of copper on the oxygen affinity of the blood of several species could be carried out to build up an overall picture of the nature of the effect of copper on respiration. Further, the uptake of oxygen at the gill surfaces could be studied in some detail using a technique which would involve the isolation of gills with the filaments intact and carrying out measurements of the oxygen consumption in different copper solutions.

Basic respiration studies should also be undertaken using a wide range of freshwater invertebrates.

7. Using autoradiographical techniques it should be possible to obtain more precise information regarding the distribution of copper within the nymphs. This would be a more sensitive technique than a histochemical one, which probably misses very small quantities of copper in the tissues. Autoradiography would also give a clearer indication of relative amounts of copper involved in different tissues.

8. It is possible that a relationship exists between size and extent of toxic effect. An investigation could therefore be undertaken in which the toxicity test could be used to investigate

size as a variable factor. Size seems to lead fairly naturally to a consideration of the different stages of the life histories of Mayfly nymphs so that work could be carried out using different instars so that the effect of copper on nymphs at different stages of development would be ascertained. This could be extended to a study including the effects of copper on the hatching of the eggs of several species.

A relationship between size and toxicity could go some way towards explaining the differential toxicity of copper not only to species of Mayfly but also in terms of a much wider range of organisms.

9. The rate of uptake should be investigated for a wider range of species both of Mayfly and other freshwater invertebrates. The effects of complicating factors such as temperature, light (as this affects diurnal activity), and pH should also be studied in terms of their effects on uptake rates. Such studies could be extended to other heavy metals. Also the uptake rates at higher concentrations than those studied here should be investigated.

10. A broad programme of research aimed at gaining information in terms of sub-lethal effects could be carried out initially with one species. Such a programme would include work on the effect of copper on certain aspects of reproduction such as the development of gonads and gameto-genesis as well as development generally. Studies in other fields could also be undertaken, and as SPRAGUE (1971) has suggested, these would include histopathology, histochemistry,

biochemistry and physiology generally. Other criteria which could be investigated could include disease resistance, and effects on production in experimental communities.

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APPENDIX

TOXICITY TEST DATA

The full tables for the toxicity test data have proved rather bulky and therefore have not been included in this volume. They have, however, been deposited, in a separate volume, in the Department of Civil Engineering, University of Newcastle upon Tyne.

TABLE 15 DATA OBTAINED FROM RESPIRATION STUDIES (Chapter 7)

B.rhodani

Time (min.)	Oxygen consumption/hr/gm			
	0	50	100	200
10	24.8 ± 19.1	8.0 ± 1.2	1.1 ± 0.5	14.3 ± 1.0
20	16.6 ± 8.1	7.5 ± 0.8	1.4 ± 1.3	11.9 ± 1.2
30	14.9 ± 7.1	7.0 ± 0.7	3.0 ± 2.0	8.2 ± 0.9
40	12.2 ± 5.0	7.0 ± 0.6	3.5 ± 1.9	8.1 ± 1.0
50	11.3 ± 3.0	6.5 ± 0.7	4.0 ± 2.1	5.6 ± 0.4
60	8.8 ± 3.0	5.7 ± 0.5	3.9 ± 1.9	4.8 ± 0.5
70	7.1 ± 4.0	5.6 ± 0.4	4.0 ± 1.4	4.1 ± 0.5
80	6.1 ± 3.0	5.6 ± 0.4	3.9 ± 1.1	3.6 ± 0.4
90	5.5 ± 3.0	5.3 ± 0.4	3.8 ± 0.5	3.2 ± 0.5
100	4.9 ± 2.6	4.8 ± 0.3	3.6 ± 0.4	2.9 ± 0.5
110	4.6 ± 2.3	4.4 ± 0.3	3.4 ± 0.2	2.6 ± 0.3
120	5.7 ± 5.4	3.9 ± 0.2	3.2 ± 0.1	2.4 ± 0.3
130	6.1 ± 4.0	3.6 ± 0.2	3.1 ± 0.0	2.2 ± 0.3
140	5.6 ± 3.5	3.4 ± 0.2	3.0 ± 0.2	2.2 ± 0.4
150	5.5 ± 3.2	3.1 ± 0.2	2.8 ± 0.2	2.1 ± 0.3
160	5.2 ± 2.9	2.9 ± 0.2	2.7 ± 0.2	1.8 ± 0.3
170	5.0 ± 2.7	2.7 ± 0.2	2.6 ± 0.2	1.7 ± 0.2
180	4.7 ± 2.5	2.5 ± 0.1	2.4 ± 0.2	1.6 ± 0.2
240	3.9 ± 1.7	1.8 ± 0.1	1.8 ± 0.2	0.9 ± 0.3
300	3.2 ± 1.3	1.4 ± 0.1	1.5 ± 0.2	0.7 ± 0.2
360	2.8 ± 1.1	1.2 ± 0.1	1.2 ± 0.1	0.5 ± 0.2
420	2.4 ± 0.9	1.0 ± 0.1	1.1 ± 0.1	0.4 ± 0.2
480	2.1 ± 0.8	0.9 ± 0.1	0.9 ± 0.1	0.4 ± 0.2
540	2.0 ± 0.6	0.8 ± 0.1	0.8 ± 0.1	0.3 ± 0.2
600	1.8 ± 0.6	0.8 ± 0.1	0.7 ± 0.1	0.2 ± 0.1
660	1.7 ± 0.6	0.6 ± 0.1	0.5 ± 0.1	0.1 ± 0.0

TABLE 16 DATA OBTAINED FROM RESPIRATION STUDIES (Chapter 7)

R.semicolorata

Time (min.)	Oxygen consumption/min/g				
	0	10	50	100	200
10	6.6 ± 2.2	9.8 ± 1.0	7.3 ± 0.0	6.1 ± 0.8	6.0 ± 1.6
20	5.5 ± 2.0	10.6 ± 0.1	7.1 ± 1.0	6.6 ± 0.8	6.7 ± 1.3
30	5.5 ± 1.9	11.1 ± 0.1		5.9 ± 0.6	6.8 ± 1.2
40	5.6 ± 1.5	10.0 ± 0.2		5.7 ± 0.6	6.3 ± 1.4
50	5.9 ± 1.5	9.6 ± 0.8		5.0 ± 0.8	5.9 ± 1.2
60	6.5 ± 1.7	9.2 ± 0.2	6.0 ± 0.8	3.5 ± 0.5	5.4 ± 1.1
70	7.8 ± 1.7	9.1 ± 0.2		3.1 ± 0.5	5.3 ± 1.0
80	7.8 ± 1.5	8.8 ± 0.2		2.9 ± 0.5	4.8 ± 1.0
90	7.3 ± 1.2	8.6 ± 0.4		2.6 ± 0.5	4.3 ± 0.9
100	6.5 ± 1.0	8.4 ± 0.9		2.4 ± 0.6	4.1 ± 0.9
110	6.2 ± 0.8	8.3 ± 0.1	4.9 ± 0.0	2.2 ± 0.6	3.9 ± 0.9
120	5.9 ± 0.6	7.8 ± 0.1		2.2 ± 0.5	3.6 ± 0.7
130	5.7 ± 0.5	7.5 ± 0.2		2.0 ± 0.5	3.4 ± 0.8
140	5.4 ± 0.4	7.3 ± 0.5		1.9 ± 0.5	3.1 ± 0.9
150	5.2 ± 0.3	7.1 ± 0.6		1.7 ± 0.5	2.9 ± 0.8
160	5.1 ± 0.3	6.8 ± 0.6		1.6 ± 0.4	2.3 ± 0.5
170	4.9 ± 0.3	6.5 ± 0.2		1.5 ± 0.5	2.1 ± 0.5
180	4.7 ± 0.3	6.2 ± 0.2	3.4 ± 0.0	1.3 ± 0.5	1.9 ± 0.3
240	3.8 ± 0.1	4.3 ± 0.2		0.6 ± 0.4	1.1 ± 0.2
300	3.5 ± 0.2	3.4 ± 0.1		0.5 ± 0.3	0.8 ± 0.3
360	3.3 ± 0.2	3.0 ± 0.1		0.4 ± 0.3	0.6 ± 0.2
420	3.4 ± 0.2	2.6 ± 0.1		0.3 ± 0.3	0.4 ± 0.1
480	3.2 ± 0.1	2.4 ± 0.1		0.2 ± 0.2	0.3 ± 0.1
540	3.0 ± 0.1	2.2 ± 0.1		0.2 ± 0.2	0.2 ± 0.1
600	2.7 ± 0.1	2.1 ± 0.1		0.2 ± 0.2	0.2 ± 0.1
660	2.4 ± 0.1	1.9 ± 0.1		0.2 ± 0.2	0.1 ± 0.1

TABLE 17 DATA OBTAINED FROM RESPIRATION STUDIES (Chapter 7)

E.venosus

Time (min.)	Oxygen consumption/hr/gm				
	0	10	20	50	100
10	3.1 ± 2.7		5.5 ± 0.9	3.8 ± 0.2	12.2 ± 0.8
20	2.3 ± 1.6	3.6 ± 1.0		1.9 ± 0.2	7.2 ± 1.9
30	4.2 ± 1.8	3.4 ± 1.0	3.0 ± 0.3	1.7 ± 0.1	7.4 ± 1.5
40	3.4 ± 1.3	3.8 ± 0.8			
50	3.1 ± 1.2	4.3 ± 0.5	2.6 ± 0.3		
60	2.6 ± 0.0	4.4 ± 0.5	2.4 ± 0.3	2.5 ± 0.2	5.5 ± 1.4
70	2.5 ± 0.9	4.3 ± 0.9	2.4 ± 0.2		
80	2.4 ± 0.9	4.1 ± 0.7	2.3 ± 0.2		
90	2.3 ± 0.0	4.1 ± 0.3	2.1 ± 0.2		
100	2.4 ± 0.9	4.1 ± 0.8	1.9 ± 0.2		
110	2.2 ± 0.9	4.2 ± 0.9	1.8 ± 0.3		
120	2.1 ± 0.8	4.1 ± 0.9	1.7 ± 0.2	3.3 ± 0.3	2.6 ± 0.4
130	2.0 ± 0.7	4.0 ± 0.9	1.5 ± 0.2		
140	2.0 ± 0.8	4.0 ± 1.9	1.5 ± 0.2		
150	2.0 ± 0.5	4.0 ± 1.0	1.4 ± 0.2		
160	2.1 ± 0.5	3.7 ± 1.0	1.2 ± 0.1		
180	1.8 ± 0.5	3.4 ± 0.9	1.1 ± 0.5	2.4 ± 0.2	1.5 ± 0.3
240	1.6 ± 0.5	2.6 ± 0.6	1.0 ± 0.2	1.8 ± 0.2	1.2 ± 0.2
300	1.4 ± 0.5	2.1 ± 0.5	0.9 ± 0.2	1.5 ± 0.1	1.0 ± 0.2
360	1.6 ± 0.8			1.2 ± 0.1	0.8 ± 0.2
420	1.4 ± 0.8	1.8 ± 0.4	0.7 ± 0.2	1.1 ± 0.0	0.6 ± 0.2
480	1.2 ± 0.7	1.7 ± 0.4	0.6 ± 0.2	1.0 ± 0.1	0.6 ± 0.2
540	1.1 ± 0.6	1.6 ± 0.3	0.5 ± 0.2	0.9 ± 0.9	0.5 ± 0.2
600	1.0 ± 0.5	1.3 ± 0.3	0.4 ± 0.2		0.4 ± 0.1
660	0.9 ± 0.4	1.1 ± 0.3	0.4 ± 0.1		0.2 ± 0.1

TABLE 18 DATA FROM ISOTOPE UPTAKE EXPERIMENTS (Chapter 8)

Values are means of six counts and are followed by standard error + or - values.

Baetis rhodani 2.7 mg/l copper (= 3 mg/l copper chloride)

Time in hr.	mc/g body wet wt.	mc water samples	mc control (no animals)
0	0.01 ± 0.0	2.4 ± 0.2	2.3 ± 0.2
2	0.9 ± 0.1	1.6 ± 0.2	1.8 ± 0.2
4	2.4 ± 0.4	1.4 ± 0.4	1.6 ± 0.5
6	3.1 ± 0.7	1.9 ± 0.7	2.0 ± 0.6
8	3.4 ± 0.8	1.6 ± 0.6	1.7 ± 0.8
12	4.1 ± 0.6	1.2 ± 0.8	2.0 ± 0.6
16	5.4 ± 0.5	0.7 ± 0.7	2.0 ± 0.7
	17.8 mg/l copper (= 20 mg/l copper chloride)		
0	0.01 ± 0.0	2.5 ± 0.1	2.1 ± 0.1
2	1.0 ± 0.1	1.5 ± 0.2	2.0 ± 0.1
4	3.4 ± 0.6	1.5 ± 0.6	1.9 ± 0.4
6	4.1 ± 0.4	1.4 ± 0.4	1.9 ± 0.4
8	4.7 ± 0.8	1.3 ± 0.5	2.0 ± 0.5
12	5.9 ± 0.6	1.0 ± 0.6	2.3 ± 0.3
16	7.6 ± 0.5	0.9 ± 0.7	2.2 ± 0.2

Rithrogena semicolorata 2.7 mg/l copper

0	0.2 ± 0.1	2.0 ± 0.2	2.1 ± 0.1
2	0.7 ± 0.1	1.5 ± 0.4	2.1 ± 0.1
4	1.0 ± 0.3	1.4 ± 0.5	2.0 ± 0.4
6	1.4 ± 0.1	1.3 ± 0.3	2.1 ± 0.4
8	1.7 ± 0.3	1.1 ± 0.3	2.7 ± 1.0
12	3.7 ± 0.6	1.1 ± 0.6	2.2 ± 0.6
16	3.7 ± 0.5	1.2 ± 0.5	2.0 ± 0.4
24	2.7 ± 0.9	1.3 ± 1.1	2.1 ± 0.6

TABLE 18 (continued)

Rithrogena semicolorata 17.8 mg/l copper

Time in hr.	mc/g body wet wt.	water samples	control (no animals)
0	0.1 ± 0.1	1.8 ± 0.1	2.0 ± 0.3
2	1.0 ± 0.1	1.7 ± 0.3	2.0 ± 0.4
4	2.3 ± 0.3	1.3 ± 0.5	2.1 ± 0.6
6	2.8 ± 0.6	1.0 ± 0.7	2.2 ± 0.4
8	3.6 ± 0.5	0.8 ± 0.5	2.2 ± 0.3
12	5.7 ± 0.4	0.6 ± 0.8	2.3 ± 0.8
16	6.8 ± 0.7	0.5 ± 1.0	2.4 ± 0.7

Ecdyonurus venosus 2.7 mg/l copper

0	0.1 ± 0.1	1.4 ± 0.3	1.1 ± 0.3
2	0.3 ± 0.3	1.0 ± 0.5	0.9 ± 0.4
4	1.1 ± 0.5	0.8 ± 0.4	1.0 ± 0.6
6	2.1 ± 0.3	0.8 ± 0.7	1.0 ± 0.6
8	2.5 ± 0.5	0.7 ± 0.5	1.0 ± 0.5
12	2.9 ± 0.4	0.6 ± 0.8	1.0 ± 0.5
16	3.9 ± 0.6	0.5 ± 0.7	0.7 ± 0.8

17.8 mg/l copper

0	0.0	1.6 ± 0.2	1.7 ± 0.3
2	0.5 ± 0.2	1.0 ± 0.3	1.7 ± 0.5
4	1.5 ± 0.4	0.7 ± 0.3	1.6 ± 0.4
6	2.2 ± 0.6	0.7 ± 0.7	1.4 ± 0.6
8	2.8 ± 0.5	0.3 ± 0.3	1.4 ± 0.4
12	4.1 ± 0.8	0.1 ± 0.2	1.2 ± 0.3
16	5.6 ± 0.9	0.1 ± 0.2	1.3 ± 0.2

TABLE 19 RATE OF UPTAKE OF Cu64 (Chapter 8)

Baetis rhodani

Time in hours	millicuries Cu64/gram (wet wt.)/hour	
	2.7 mg/1 Cu	17.8 mg/1 Cu
2	0.45 ± 0.05	0.50 ± 0.05
4	0.60 ± 0.1	0.85 ± 0.15
6	0.50 ± 0.12	0.68 ± 0.06
8	0.42 ± 0.10	0.65 ± 0.10
12	0.34 ± 0.05	0.49 ± 0.05
16	0.33 ± 0.03	0.47 ± 0.03

Rithrogena semicolorata

Time in hours	millicuries Cu64/gram (wet wt.)/hour	
	2.7 mg/1 Cu	17.8 mg/1 Cu
2	0.35 ± 0.05	0.50 ± 0.05
4	0.25 ± 0.08	0.58 ± 0.08
6	0.23 ± 0.02	0.47 ± 0.10
8	0.21 ± 0.04	0.45 ± 0.06
12	0.31 ± 0.05	0.47 ± 0.03
16	0.23 ± 0.03	0.43 ± 0.04
24	0.11 ± 0.06	

Ecyonurus venosus

Time in hours	millicuries Cu64/gram (wet wt.)/hour	
	2.7 mg/1 Cu	17.8 mg/1 Cu
2	0.15 ± 0.15	0.25 ± 0.10
4	0.28 ± 0.13	0.38 ± 0.10
6	0.35 ± 0.05	0.37 ± 0.10
8	0.31 ± 0.06	0.35 ± 0.06
12	0.24 ± 0.03	0.34 ± 0.07
16	0.24 ± 0.04	0.35 ± 0.06