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COPPER TOXICITY MECHANISMS IN ALGAL SYSTEMS AND ISOLATED CHLOROPLASTS.

by

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ABSTRACT

Using algal systems and isolated pea chloroplasts, a progression of experiments was conducted, whereby work initially looked at copper's effects on algal growth and then followed a more biochemical approach which tried to elucidate actual sites of copper inhibition.

The growth of both Chlorella pyrenoidosa and Sargassum muticum cultures was inhibited by copper. Sargassum was particularly sensitive, but Chlorella exhibited a more graded response to increasing metal levels. Toxicity increased with decreasing chelator presence.

Uptake experiments showed metal accumulation in living and dead tissues and Chlorella pigment extractions demonstrated reduced chlorophyll levels and a:b ratios following metal treatments of living cells. Chlorella photosynthetic rates fell with increasing copper levels, but respiration could be stimulated, perhaps because membrane permeability changes caused increased substrate availability.

In Chloroplasts, overall electron transport rates through the whole photosynthetic system, and through photosystem one (PS I) and photosystem two (PS II) assayed seperately, were reduced with increasing copper doses. PS II was the most sensitive of the two and uncoupled rates were more susceptible to inhibition than were coupled. Dark preincubation reduced metal interference.

A dual effect of copper emerged:inhibition of photosynthetic electron flow and an uncoupling action. At low concentrations, the uncoupling action was detected as a stimulation in the electron flow rate and an increase in the rate of decay of the flash induced electric field, indicating an absorption change at 518 nm. An increase in the concentration of chlorophyll present resulted in greater apparent inhibition of coupled, but a reduced effect on uncoupled rates.

Chlorophyll fluorescence experiments with Chlorella showed that copper inhibits electron flow and evidence was accumulated that the site of inhibition was in the region of PS II. Time of exposure to the metal was important, particularly with low concentrations. In the case of chloroplasts, both tris-washed and untreated isolated

thylakoid membranes were used. It was established by chlorophyll fluorescence monitoring, that copper inhibits PS II by blocking electron flow from water to the reaction centre P680. Treatment with PS II electron donors did not overcome the inhibition, although they were capable of by-passing the tris inhibition site. It is concluded that the main action of copper is at a site close to the PS II reaction centre, although with Chlorella some restoration of PS II activity was noted on addition of donors.

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CHAPTER I

INTRODUCTION

1. INTRODUCTION

1. General introduction and reasons for interest in metal/plant interactions.

There is an extensive literature devoted to the various effects of heavy metals on plants. This literature reflects the interest in metal/plant interactions that has arisen because of the expansion of industrial and agricultural processes that, wittingly or otherwise, have increased metal presence in the environment. Thus, levels of metals such as zinc, cadmium, copper, aluminium and lead have risen in some situations because of pollution from industrial plants, the burning of fossil fuels, mineral extraction and refining activity, as well as the case of direct, intentional application for purposes of, for example, weed or pest control.

In order to minimize the various demaging and harmful effects that can arise within an ecosystem because of some of these activities, it has become clear that a deeper understanding is required of the processes and mechanisms that are involved when plants and algae react unfavourably in response to metal contamination. By working to increase the knowledge of the way in which metals act to give specific effects, one aims to more assuredly be able to predict and foresee the probable ecological outcome of a particular course of action. By undertaking laboratory studies on specific aspects of metal involvement in plant growth, development or metabolism, one can gradually establish a pool of knowledge that can be utilized in the context of metal involvement in the field situation. The aquatic environment is often at particular risk because it can receive runoff from adjacent contaminated land. For this reason, much work has involved studies with algae and other aquatic plants.

That heavy metals, rather arbitrarily defined as being metals of density greater than 5 g cm^{-3} (PASSOW et al., 1961), have specific and definite actions on various physiological processes is also of intrinsic interest. A study that aimed to be exhaustive and cover the toxicological effects of all, or even several, heavy metals would clearly be of great length and this current work has therefore aimed to investigate one metal only-copper. An attempt has been made to combine an overall investigation of some of the grosser effects of copper on algal growth with a more physiologically biased approach that tried to go some way towards pinpointing some of the specific sites at which copper was exerting its influence.

1.1 Two main approaches to the study of copper toxicology: By looking at copper's immediate toxicological effects and by studying plants that can cope with a metal incursion.

Man's activities have, in several different ways, facilitated the movement of metals into the environment in a form available to plants. It is true that plant communities may also be affected by naturally occuring ore deposits and particular species may be used as indicators of underlying mineralisations. For example, ANTONOVICS et al., (1971) list thirty-one species that have been cited as being indicative of the presence of copper. However, in the context of copper toxicology, these plants are perhaps less interesting than are those affected by extraneous copper sources because those occuring naturally at the site of an anomaly can grow successfully only because they have developed resistance or tolerance mechanisms to the metal. The effects of the copper in these plants can therefore be masked, whereas in a plant affected by a relatively recent metal intrusion, toxicological effects can sometimes be studied.

There have, therefore, perhaps been two main directions followed by the research that has aimed to investigate the impact of copper on the plant kingdom; one of these has been to study the way that some plant species have come to cope with increased metal levels, mainly by the perhaps relatively rapid evolution of various tolerance mechanisms. The other approach has been to try to elucidate the more short term effects of copper on various aspects of the plants metabolism; with this approach, one is attempting to discover how the copper is actually exerting its influence, at what concentrations, at what exact points in the biochemical pathways and, if it has more than one effect, what is the order of susceptibility?

It is mainly by asking these latter type of questions, particularly in the context of algal communities, that this current work has attempted a contribution to the overall picture, but because there is considerable overlap in the approaches used in the literature, and for purposes of completeness and continuity, it has been desirable to include reference to some of the other aspects studied by other authors.

1.2 Sources of environmental copper contamination:

Perhaps the most common source of environmental copper contamination is from metal smelters. These may be specifically geared to copper extraction or the copper may simply be a contaminant of the mine tailings. Thus, FOY et al (1978) have said that, on mine spoils and other wasteland, excess copper, among other metals, can limit plant growth, particularly in acid soils (where copper would be more likely to be present in a form available to plants), but also sometimes at more neutral pH. ANTONOVICS et al (1971) also list possible sources of metal pollution and include reference to unproductive ores, deposited on the surface, and seepage from wastes, as being important agents of contamination.

Mine wastes, and more particularly the effluvia emitted from smelter chimneys, can give rise to aerial forms of metal pollution, including copper. Thus, HEMPHILL and CLEVENGER (1979) found elevated levels of Pb, Zn, Cu and Cd in white oak leaves growing a mile away from the site of lead smelters in Missouri, while Wu and BRADSHAW (1972) found that copper was the "chief constituent of the dust particles that have been emitted" from the metal refining industry in South Lancashire and this had given rise to soil contamination of up to 400 ppm copper with complete vegetation destruction in some areas. ALLEN and STEINNES (1979) gave evidence for the heavy metal contamination of southern and coastal regions of Norway. They submitted the theory that this was the result of the long-range transport of pollutants from sources to the south and south west of the country. RANDALL et al (1979) have also claimed that at least seven heavy metals, including copper, are detectable in the storm water runoff in the Washington DC area. They said that the dominant source of these contaminants were heavy metals washed out of the atmosphere. In similar work, HOWELL (1979) monitored the heavy metal composition of runoff from Californian highways. He found that the growth of algal colonies in lakes receiving the runoff could be severely inhibited by this input.

Mining activities can lead to metal contamination of other types of water environment. A study by VIVIAN and MASSIE (1977) showed that high trace metal levels, including copper, could be found in rivers draining the Swansea valley in South Wales. These elevated levels were the result of the weathering, erosion and runoff from waste materials left by smelting industries. STOKES et al (1973) found

similarly high metal levels, particularly of copper, in lake waters in the Sudbury smelting region of Ontario, Canada. Similarly, PINE (1979)found direct relationships between "local human activities" and the heavy metal contents of submerged vascular plants and estuarine sediments in Chesapeake Bay. BEAUFORD et al (1977) demonstrated that plant surfaces themselves can release metals into the atmosphere. This is a fact that should perhaps be borne in mind when considering low levels of atmospheric contamination.

Heavy metals, and specifically copper, have also been intentionally added to the environment for some considerable time. Because of their recognized high toxicity, they have been incorporated into fungicides, pesticides, algicides and disinfectants. (ANTONOVICS et al, 1971). By using these compounds without sufficient regard for their long term environmental impact, copper toxicities can appear in plants on previously-productive cropland (BROWN and JONES, 1975). LEEPER (1972) has stated that "if we add materials containing foreign metals to a soil, we are making a permanent change. They may be held harmless over many years of periodic additions, but as the years go by and the total concentration increases, the possible uses of the land decline". For example, although copper is essential to "all living organisms" (WILLIAMS, 1967), the presence of too much of it can lead to an inbalance in other essential nutrients; FOY et al (1978) quote that "Fe deficiency chlorosis of citrus seedlings in Florida has been partially attributed to the accumulation of excess copper resulting from repeated spray and fertilizer applications". DELAS (1963) made a similar point in stating that "copper reaching the soil through fungicides may accumulate to a concentration which is toxic to plants and may become manifest through a reduction in plant development".

Heavy metals may be concentratedin sewage (BERRY, 1976) and, therefore, if this is used to fertilize farmland, attention must be paid to their potential further concentration by crop plants. BAKER (1974) pointed out that "the practice of adding up to 250 ppm of copper as copper sulphate to rations for swine and poultry could provide 1.5 to 3 ppm of copper to surface soil per year on land where manure is used as a source of nitrogen for corn ". BERROW and BURRIDGE (1979) reinforce this point by demonstrating a marked persistence of high levels of readily extractable Cd, Cr, Cu, Ni and Zn in soil following a sewage treatment and herbage species sampled nearly ten

years after the sludge application contained elevated levels of Cu, Ni and Zn. PURVES (1979) pinpointed the same problem and suggested that the levels of permissable metal additions to farmlands should be revised downwards. He also said that the standards should take more account of the various toxicities of different metals, the relative ease of uptake by plants etc.

Perhaps the most widespread and effective use of copper in the environment has been as an algicide, a role in which it is arguably more effective than other heavy metals. This metal has been used to control algae in reservoirs, drainage ditches, swimming pools and lagoons. In earlier times, copper was probably used simply because it was known to be effective (MOORE and KELLERMAN, 1904), but gradually a wealth of knowledge has been established so that the dose level and form of application can be more suitably defined for a given situation (PRESCOTT, 1948: AMERICAN WATER WORKS ASSOCIATION, LTD., 1950: DERBY and TOWNSEND, 1953 : BARTSCH, 1954).

Copper has been incorporated into the anti-fouling paints used to keep the undersides of ships free of algae and molluscs and, in some ways as a follow-up to this type of application, in more recent years, work has been conducted to incorporate copper into so-called "Control Slow Release Glasses" (STANDARD TELECOMMUNICATION LABORATORIES, 1976). These are commercial products in which copper, for example, is held within a structure of other non-toxicological compounds (chiefly P_2O_5). The final form of the "glass" can be a powder, fibre or piece of rock-like material which, when placed in water, will gradually, and at a known rate, dissolve and release the copper toxin into the aqueous environment, there to control algae, molluscs, mosquitoes or whatever. The advantage of these compounds is that, by varying the relative proportions of the constituents, one can control the rate of release of metal and the duration of its period of potential effectivity. In this way, it is possible to produce a glass that can give a steady dose for periods ranging from minutes to years. This situation contrasts with the more conventional methods of application where one can give a high critical copper dose (usually as the sulphate) which thereafter declines, perhaps very quickly, because the metal can be effectively "lost" due to precipitation as insoluble forms, chelation by organic matter, complexing with other groups present or simple runoff and seepage (McINTOSH, 1975). It is argued that, for some applications, a constant level of

copper over a prolonged period is more likely to prove an effective poison than is a high initial dose that soon declines, perhaps allowing the organism to recover.

1.3 The general reactivity of copper in biological system and the relative toxicities of various forms.

Heavy metals have a high potential reactivity in biological systems generally. This is because they can combine with many organic molecules and can link to ligands of many types, including those present in proteins (PASSOW et al, 1961). They are thus strong enzyme inhibitors; they can form stronger bonds with some of these enzymes than can the lighter metals usually present, and so the inhibition is often competitive. It has often been said that heavy metal binding by biological material is strong but not specific with regard to either the heavy metal or the ligand (GURD and WILCOX, 1956). Perhaps the only rules that can be established are concerned with the order of affinities. Copper often emerges with one of the highest of these affinities for ligands commonly found in biological cells.

In any living cell, at least the following ligands can be expected to be present: $-OH$, $-COOH$, $-PO_3H_2$, $-SH$ and $-NH_2$. Knowing the relative affinities of each heavy metal to various ligands enables one to loosely predict susceptible sites. For example, heavy metals are bound by amines or simple amino-acids in the following order of decreasing affinity:
$Hg^{2+} > Cu^{2+} > Ni^{2+} > Pb^{2+} > Zn^{2+} > Co^{2+} > Cd^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+} > Ba^{2+}$, whereas the affinity for $-COO^-$ group is as follows:
$Cu^{2+} > Ni^{2+} > Zn^{2+} > Co^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+} > Ba^{2+}$.

As copper will bind simultaneously at sensitive and insensitive sites, the toxicological effect may be produced by a relatively small proportion of the applied metal. There are, therefore, often problems in toxicological experiments as it can be difficult to be sure about the level of copper that is inhibitory for a given system. A proportion of the applied metal may be precipitated as insoluble carbonates or hydroxides, bound by chelators such as ethylenediaminetetra-acetic acid (EDTA), perhaps present in the growth medium, or even be adsorbed by the surface of experimental vessels, the degree and strength of the adsorption varying with other factors including the nature of the surface. (ELLIOTT et al, 1979). Even then,

of the proportion of copper that reaches the plant, only a further percentage reaches sensitive sites.

Plant cellular structure governs the accessibility of these sites and of important ligands. The cell wall is the first barrier to penetration, but the cell membrane is almost invariably the first physiologically-sensitive structure to be encountered by copper. This means that sensitive ligands situated on cell membranes are particularly susceptible to copper damage. PASSOW et al (1961) have said that "the universal reactivity of the metals and the ubiquity of metal-binding ligands lead one to expect that biological factors, especially those governing the accessibility of the various cellular receptors, play a dominant role in metal toxicology. The cell membrane is the first and most important site of action of metals". This follows a near-identical statement from Rothstein (1959) that "the general pharmacology of heavy metals is therefore largely concerned with pathological changes of function associated with the cell membrane".

This prime susceptibility of membranes had been recognised for sometime. As far back as 1930, ADAM quoted that "cell membranes contain large quantities of lipids, mainly phosphatides. It is well known that very small amounts of heavy metals produce appreciable changes of surface tension and surface change of lipid films. Alteration of these variables may be expected to lead to marked changes of (membrane) permeability and metabolic activities of surface enzymes".

McBRIEN and HASSALL (1965) also concentrated on the action of copper on membrane permeability. Using cells of Chlorella Vulgaris, they found that toxic doses of copper caused the release of potassium into the medium in amounts not related stoichiometrically to the amount of copper bound to the membrane. Ion exchange was therefore not the mechanism involved and they said that potassium was released "due to a graded respose of a barrier, normally of low permeability, to increasing amounts of bound copper. This increase in the permeability of the cells is considered to be the primary toxic effect of copper". DE FILIPPIS (1979a) obtained similar results using several metal chlorides (including $CuCl_2$) on Chlorella species and broadly consistent results were also quoted by SHIEH and BARBER (1973) using mercuric chloride and by PUCKETT (1976) using copper on lichens.

In plant cells, therefore, there is usually a time-scale series of events from the outside to the inside of the cell. The first reactions of copper are with ligands of the cell surface, causing associated disturbances of membrane function. This is followed by metal penetration of the cell, whereupon secondary additive effects are likely to become manifest. It is usually of importance to try and study the primary effects on any system that one may have interest in because copper's near-ubiquitous reactivity means that many other effects are likely to follow on and any one of these may affect one's initial point of interest via secondary processes.

The form in which copper is supplied to the plant is also of great importance to the subsequent development of a toxic reaction, if any. STEEMANN-NIELSEN and WIUM-ANDERSON (1970) stated that copper, in ionic form, and at the concentrations normally found in natural waters, was very poisonous to the photosynthesis and growth of algae. It was concluded, therefore, that since algae usually survive and thrive in nature, the copper could not be present as ions, but as complexes linked to organic material such as polypeptides. GACHTER et al (1973) agreed that "natural waters have the property to mask added Cu^{2+} ions", and MARQUENIE-VAN DER WERFF (1979) demonstrated that the presence of EDTA reduces the uptake of zinc into metal-treated Holcus lanatus plants, as does humic acid, a natural complexing agent found in peaty soil.

AUSTENFELD (1979) reported a similar result for Bush beans, Phaseolus vulgaris, where the uptake of nickel was reduced by about a factor of 5 when Ni-EDTA, as opposed to $NiSO_4$, was applied. RAY et al (1979) agreed that "complexed and chelated heavy metals are less toxic to aquatic organisms".

DEKOCK and MITCHELL (1957) worked with mustard and tomato plants and showed that copper, along with other divalent cations, was more readily taken up when present in solution in ionic form than when chelated, particularly with EDTA. They hypothesized that one possible explanation was that " the charge on the chelated molecule is one factor controlling absorption by the root, those with no charge or a single negative charge being taken up while complexes with two charges are not". This point has been made by other authers (COOMBES et al, 1977 and 1978). ANDREW(1975) concluded that the cupric ion, Cu^{2+}, is the major toxic form of copper; in tests with Fathead minnows and Daphnia magna, copper complexes,

such as soluble carbonate, were found to be much less harmful than was ionic copper.

Various other chelating agents have been in vestigated by different authors, but always reduced toxicity is the result of copper-complexing. Amongst these compounds so studied have been EDTA (TABATA and NISHIKAWA, 1969), nitrilotriacetate (BIESINGER et al, 1974; SHAW and BROWN, 1974) and humic acid (MARQUENIE-van der WERFF, 1979). NISHIKAWA and TABATA (1969) also demonstrated that the reduction in toxicity is proportional to the stability constants of the chelators.

Chelated forms of copper are therefore relatively non-toxic, this reduction in toxicity being brought about by a lowering of cupric ion activity. Working with the diatom Thalassiosira pseudonana, SUNDA and GUILLARD (1976) confirmed the point by stating that "growth rate inhibition and copper content of cells are related to cupric ion activity and not to total copper concentration. Although working with maize seedlings, and using aluminium as the experimental metal, BARTLETT and RIEGO (1972) came to an identical conclusion when comparing the effects of ionic and citrate or EDTA-complexed metal forms. ANDREW (1976) followed up his previous work by looking at copper complexing with carbonate-bicarbonate, orthophosphate and pyrophosphate. He too was convinced that it was ionic, and not total, copper that was directly correlated with toxicity.

One of the few papers to present data contrary to this idea was that by FITZGERALD and FAUST (1963) who reported that toxicity was independent of the sources of copper used and that precipitated copper was as effective as copper in solution. This surprising result may be partially explained by an idea quoted by MOORE and KELLERMAN (1904) which stated that insoluble copper salts, such as the hydrate, carbonate and phosphate, are toxic only if in actual contact with the cells. In a culture situation, the precipitate and cells would be intimately mixed and the copper might therefore exert a toxicological action, whereas, in a natural environment, the precipitate could fall to a depth below that of algal growth and so be effectively removed from the system.

1.4 Resistance and tolerance mechanisms to copper in plants.

Having summarised some of the routes by which copper enters the environment

and some of the wider aspects of its biological reactivity, it will be of interest to consider some of the mechanisms employed by plants, in some instances, to cope with the metal incursion. ANTONOVICS et al (1971) made the point that it is not easy to exactly define what is meant by the term "metal tolerant"; they said that it applies in two main senses. In one case, it applies to species occuring in an area of metal contamination from which other species appear to be excluded. The other case is more specific as it deals with a species, normally intolerant, but which has an ability to evolve tolerant races.

As already noted, there are many instances recorded of plants, including so-called indicator species, being able to grow successfully on soil with a high copper content. These plants must possess some means whereby either the copper is kept away from sensitive sites in the plant or the plant is so adapted that the sensitive sites are less affected by copper than is the case in other species. Such plants have been recognised as being useful tools in mineral prospecting. CANNON (1960) and BOLLARD and BUTLER (1966) said that these plants can be employed in threeways: (a) by mapping the distribution of particular species (indicator plants) most affected by the minerals sought, (b) by observation of physiological and morphological changes in plants growing near ore bodies and (c) by detection of differences in plant composition.

A point always to be borne in mind is that it is the level of available copper in the soil that is of relevance in these studies; the lower the soil pH, the more likely is the copper to be available for uptake (WARREN and DELAVAULT, 1949; SIMON, 1978). Thus, PAGE et al (1979) said that detrimental effects on plant yield are more likely to occur as a result of heavy metal accumulation after sewage applications when a pH of 5.5 obtains rather than in neutral calcareous soils. There are also many other relevant factors. The presence of more than one metal can be a complication, and the general nutrient status is of importance. HILTON (1967) and SMITH and BRADSHAW (1970) both demonstrated that the addition of complete fertilizer greatly improves the growth of plants, both native and foreign, on contaminated mine soils. The point is that the degree of tolerance, of success of a plant growing in a metalliferous area often depends on more than just its ability to cope with the metal. The ability of organic matter in the soil to form stable

complexes with metal ions (HODGSON et al, 1966) can effectively reduce copper levels; DYKEMAN (1966) recorded concentrations of 7% copper in peat of a copper swamp and yet it appeared to have no toxic effects, the over-lying vegetation being typical for the area.

The evolution of heavy metal tolerance is well documented, and if the potential for its production exists within a species, it seems to be a relatively rapid process, taking perhaps twenty years or less to establish a tolerant race (WU and BRADSHAW, 1972). GARTSIDE and McNIELLY (1974) presented evidence that certain species, e.g. Agrostis tenuis, possessed the ability to evolve copper tolerance, but that this was less true, if at all, for Lolium Perenne.

One reported method of plant tolerance to copper is that of excluding the metal from physiologically-sensitive areas. This often means letting only small amounts through to aerial parts of the plant. NICHOLLS et al (1965) showed that over a fairly low range of soil copper levels, the amount in the plant remained at a low and consistent level, but that above a certain soil copper content, specific for the species, the amount in aerial parts increased abruptly and soon the plant ceased to grow. In this example, there was, therefore, a limit to the copper tolerance, but this can be less true for other examples. The African plant Becium homblei translocates excess copper, absorbed during the growing season, to the leaves where it is stored in cell walls. The leaves are regularly burnt off by bush fires in the dry season. REILLY and STONE (1971) have put forward this sequence of events as having been important to the evolution of the species. HOWARD-WILLIAMS (1972) took the idea further, but thought that the mechanism would work even without the fires because the leaves die off annually anyway.

Following the paper of NICHOLLS et al (1965), WU and co-authors (1975a) carried out further work with copper-tolerant and non-tolerant clones of Agrostis stolonifera, obtained from a control environment and from the vicinity of a Lancashire copper smelter. They found that, when the plants were grown in a copper-containing nutrient medium, the roots accumulated more copper than did the shoots. At external copper concentrations above 10 μM, the copper moved into the leaves of the non-tolerant more readily than it did into those of the tolerant clone. They also found

that the non-tolerant roots were more sensitive to respiratory inhibition by copper than were the tolerant ones and that L-malate dehydrogenase extracts, prepared from non-tolerant roots pretreated with copper, showed reduced activity when compared to similar extracts from tolerant individuals. It was suggested that this was due to an "inhibition of protein synthesis in the roots of the non-tolerant clone". The tolerant plants were therefore here operating some form of internal exclusion mechanism, restricting the amounts of copper that could get to the relatively-sensitive aerial shoots.

In a further paper, WU and ANTONOVICS (1975b) showed that, in Agrostis stolonifera plants tolerant to both copper and zinc, the uptake of one metal had no effect on the uptake of the other. However, using the parameter of root elongation, interaction between the effects of the two metals was shown and the conclusion drawn that the mechanisms of copper and zinc tolerance were "independent even when they act in the same individual". This observation fits well with previous research which showed that metal tolerance seemed to be largely, if not wholly, metal specific and that tolerance to one metal afforded no protection against another (STOKES, 1975: GREGORY and BRADSHAW, 1965). EHINGER and PARKER (1979) reported a greater tolerance to copper in urban populations of the grass Andropogon scoparius than in plants from a rural environment, but the plants showed no differences in their reactions to zinc.

Thus, multiple tolerance to two or more metals is usually associated with the presence of elevated levels of all the relevant metals in the soil. However, COX et al (1979a and b) have presented evidence to show that the development of tolerance to one metal can coincidentally increase tolerance to other metals, not present at high levels. They found that Deschampsia cespitosa plants tolerant to Ni, Cu and Al also had increased resistance to lead and zinc. ALLEN and SHEPPERD (1971) reported a similar phenomenon in Cu-tolerant Mimmulus guttatus which also had elevated tolerances to Ni and Zn. COX (1979b) makes the point that there are some grass genotypes that may be even more versatile than was first thought and which may therefore be of great use in the revegetation of metal contaminated sites.

Although it has been suggested that one method of copper resistance is to prevent this metal's free access to sensitive sites, it has gradually become clear that,

although particular parts of the plant may be spared from copper's effects, it is not usually the case that a tolerant plant takes up less total metal than a sensitive one. Indeed, the amount of uptake is often equal to, or greater than, that in a non-tolerant plant (WU et al, 1975a). The tolerant plant is therefore successful by chemically detoxifying the metal or by shunting it to a site, such as cell walls, where it can do relatively little harm (BROWN and HOUSE, 1978). Actual metal exclusion from the entire plant has therefore been considered as a rare mechanism of tolerance, but an example of its use in the green alga Chlorella vulgaris was quoted in 1977 by FOSTER. This author found that a relatively copper tolerant strain was not only less sensitive to growth inhibition by copper, but that the uptake of metal by the cells was also far less than in a control strain. A similar result was quoted for the alga Ectocarpus siliculosus by HALL et al (1979), but here the mechanism suggested for the decreased uptake was a change in the cell membrane.

In work with the bryophyte Solenostoma crenulatum uptake of supplied copper was shown to be the same in plants taken from the environs of a lead mine as from a copper mine (BROWN and HOUSE, 1978). That, despite this normal level of copper uptake, the copper mine strain was more tolerant to this metal than the lead mine "control plants" was shown by the fact that the bryophyte accustomed to the lead environment showed the greater potassium leakage and photosynthetic inhibition of the two, when copper treated. It appeared that the copper mine plants suffered less membrane damage, as a response to a copper treatment, than did the lead mine plants. This idea effectively refuted the theory put forward by BROWN and BARBER (1972) that similar uptake implied similar tolerance.

The phenomenon of copper causing potassium leakage also figured in the work of WAINWRIGHT and WOOLHOUSE (1977) who demonstrated this effect on roots of Agrostis and was mentioned by McBRIEN and HASSALL (1965) and SAKAGUCHI et al (1977) in studies with Chlorella.

The idea that some plant cells can compartmentalise copper into sites where it can cause little damage has attracted attention from several authors. Working with yeast cells, NAIKI (1957a) showed that much of the copper absorbed by tolerant cells was precipitated as copper sulphide, a relatively harmless form. ASHIDA et al (1963) showed by electron microscopy that large amounts of this copper sulphide are deposited under the cell walls.

It has also become obvious that a portion of the copper in the resistant strain is to found associated with proteinaceous material (NAIKI, 1957b). MURAYAMA (1957 and 1972) was able to demonstrate a quantitative, although not qualitative, difference between the protein composition of copper-resistant cells and that of the parent strain. The theory is that copper complexed with protein in this way is less toxic to the organism.

Further work was conducted by NAIKI and YAMAGATA in 1976 when they succeeded in extracting three types of copper-binding proteins from copper-resistant Saccharomyces cerevisiae. NAIKI(1961) reported results showing that, although resistant cells produce more hydrogen sulphide than the parent strain, they produce very little of it during the logarithmic phase of growth, even though the cells are accumulating appreciable amounts of copper. NAIKI and YAMAGATA (1976) concluded that other copper-binding substances, the proteinaceous materials, also played a significant role in yeast's copper tolerance. This overall picture fits in well with YANAGISHIMA (1957) who described a mutant, derived from a copper-resistant strain, that had no high sulphide-producing activity and yet was tolerant while still accumulating copper significantly. This type of evidence led to the generalisation by NAIKI and YAMAGATA (1976) that " the production of copper-binding components is a physiological property common to some yeast species which, when grown in a copper-toxicated environment, quickly produce them to reduce injury".

SILVERBERG et al (1976) worked with the green alga Scenedesmus and were able to demonstrate, by electron microscopy, that, as a response to copper sulphate treatment, the cells produced nuclear inclusions in the form of "central dense-core complexes". Transmission electron microscope X-ray analyses of these structures provided evidence that they contained copper. They suggested that the inclusions are the result of a cellular detoxifying mechanism, whereby copper is complexed, presumably by protein ligands. It was stated that, by this method, cytoplasmic organelles would be "to a certain extent protected from the toxic effects of copper".

Although so far only demonstrated for mercury, an interesting mechanism operated by Chlorella cells for reducing the extent of poisoning by this metal was shown by BEN-BASSAT and MAYER (1975). They said that the presence of Chlorella

cells caused a rapid decrease in mercury levels in nutrient media, the rate of decrease depending on the strength of the algal inoculum. It was hypothesized that the levels fall initially as a result of uptake by the cells. Increased volatilization of mercury to the atmosphere then begins to occur because of a reaction between the mercury and a compound produced by the cells. This reaction produces a mercuric form more volatile and less toxic or permeable than the original mercury. Thus, the overall level of mercury in solution is reduced by the cells exerting an influence to increase its volatility. In 1977, BEN-BASSAT and MAYER gave evidence for the "presence of low molecular weight, mercury reducing factors in Chlorella cells". This can therefore serve to demonstrate that Chlorella, the object of many heavy metal tolerance and toxicity studies, is capable of exhibiting more than one way of coping with a metal incursion.

STOKES et al (1973) isolated algae (Scenedesmus and Chlorella) from copper-contaminated lakes in Ontario, Canada, and discovered them to be metal-tolerant when compared to control laboratory strains. Further work (STOKES, 1975) led him to hypothesize that the tolerant forms were resistant because of the presence of a protein component that could complex the metal. In one algal strain, the nucleus appeared to be the site of copper accumulation.

The research of STOKES had started from the observation that planktonic algae survived in lakes known to be metal-contaminated. Other authors have started from the same standpoint and been able to show tolerance in algae subjected to copper pollution. Thus, MYSLIK and HUTCHINSON (1971) also showed that algal flora, isolated from heavily copper-polluted lakes in the Sudbury smelting region of Canada, showed a high degree of copper tolerance, while RUSSELL and MORRIS (1970) demonstrated that Ectocarpus siliculosus plants collected from a ship's hull were more tolerant by a factor of ten to dissolved copper than were control plants from the shore. The explanation put forward was that the ship's hull had been treated with a copper-based anti-fouling preparation and the alga had thus been able to develop resistance over a period of time. SIMERAY and DELCOURT (1979) suggested that Cu-treated Euglena gracilis cells could develop resistance towards this metal, although tolerance to cobalt and zinc was not found.

One can only assume that some of these more descriptive observations might be

based on tolerance mechanisms similar to those mentioned above.

1.5 Uptake and accumulation of copper and associated effects on plant growth

Having covered some of the more general effects of copper ions in biochemical systems, and made reference to the relative toxicities of various copper forms, it will be of use to now consider some of the investigations that have looked at copper uptake and accumulation in plants. This aspect has close links with the effects that copper can have on overall plant growth and success.

In higher plants, the accumulation of cations in roots is a result of two processes: (a) binding of ions to charged sites within the root apoplasts due to electrostatic forces, and (b) passage of ions across cell membranes into the symplast. It is the latter process which is of greater interest, as it represents the biologically-available fraction of the total root cation pool (HARRISON et al, 1979).

However, much of the work conducted on copper uptake and accumulation has been carried out using micro-organisms, particularly unicellar algae. KNAUSS and PORTER (1954) found that the amount of copper absorption by Chlorella cells was directly proportional to the concentration of that element in the nutrient solution. It is perhaps timely to note at this point that copper has been clearly established to be an essential trace element for algae. WALKER (1953) reported the need for copper at a level of 30 $\mu g\ l^{-1}$ for Chlorella pyrenoidosa if optimal growth was to be achieved. This value was confirmed by MANAHAN and SMITH (1973) for Chlorella vulgaris and a level of 40 $\mu g\ l^{-1}$ put forward for Oocystis marssonii.

BASZYNSKI et al (1978) looked at copper deficiency effects on the photosynthetic activity of higher plants. They found that chlorophyll synthesis was depressed by the deficiency and that the Photosystem One (PSI) activity of the copper-deficient chloroplasts, measured in terms of O_2 uptake (using methyl viologen as an artificial electron acceptor in the Mehler reaction) and the photoreduction of NADP, was much lower than that of the controls. Photosystem Two (PS II) activity was affected to a lesser extent. Plastocyanin, a Cu-containing protein and a component of the electron transport chain between PS II and PS I, and total copper content in deficient chloroplasts decreased over 50% in comparison with controls. These observations led the authors

to believe that plastocyanin was the only site of copper occurence in the electron transport chain. This was confirmed by a "distinctly significant decrease in the plastocyanin content of the chloroplasts of both oat and spinach" – when a copper deficiency was engineered in the plants.

The authors concluded the above paper by saying that "it should be pointed out that copper deficiency affects the light reactions mainly by reducing the plastocyanin content of the chloroplasts. This results in a decrease in photosynthetic activities connected with PS I and in a disintegration of thylakoid membranes, mainly stroma lamellae. The facts found do not exclude the possibility of the inhibition of other stages of photosynthesis".

A paper by DeFILIPPIS (1979a) looked at the effect of heavy metal compounds on the permeability of Chlorella cells. One of the most interesting points to emerge from this work was that the uptake of copper proceeds in two phases. Evidence for this is the fact that a metal treatment results in an increase in membrane permeability, enabling potassium to leak out; 90% of the total leakage is independent of light or dark conditions. However, light increases the total efflux while a pure nitrogen atmosphere, CO_2 - free environment or treatment with 3- (3',4'-dichlorophenyl) -1, 1- dimethylurea (DCMU) all reduce it. He therefore said that there is a second component to the metal uptake which "appears to require energy from photosynthesis". He thought that this bi-phasic uptake had not been quoted before because the smaller, energy - dependent component would usually have been lost amongst the light-independent uptake.

WAKATSUKI et al (1979) reported a biphasic absorption of copper into yeast cells, while MARQUENIE-VAN DER WERFF and ERNST (1979) described a similar system for the uptake of copper (and zinc) into the aquatic plant Elodea nuttallii. The authors said that the kinetics of the uptake suggested on active mechanism.

HORIKOSHI et al (1977), using Chlorella vulgaris, found that scalded (i.e. dead) cells took up larger amounts of copper than did control cells. The accumulation of metal by the scalded cells was faster than in controls and the concentration factor was also greater. Because more of the absorbed copper was removable, by a subsequent EDTA wash, in heated than in control cells, it was concluded that the metal was associated with weaker ligands in the former case, i.e. that the distribution of copper

in the live and dead cells was different. A greater uptake of cadmium by dead cells, as compared to controls, has also been described by MANG and TROMBALLA (1978).

HORIKOSHI et al (1977) compared the concentration factors for various heavy metals in the dead cells and produced the following decreasing order: $Cu^{2+} \gg Cr^{3+} > Cd^{2+} > Ni^{2+} > Zn^{2+} > Co^{2+} > Mn^{2+}$. A very similar order was quoted by SAKAGUCHI et al (1977) for the relative toxicities to Chlorella, i.e. $Hg^{2+} > Cu^{2+} > Cd^{2+} > Cr^{3+} > Fe^{2+} > Ni^{2+} > Zn^{2+} > Co^{2+} > Mn^{2+}$. This paper stated that the amount of copper absorbed by Chlorella increased in direct proportion to the amount of copper ion present in the medium and that the uptake increased rapidly over the first 120 minutes, but then little increased with the further passage of time. Loss of cell potassium was again noted as a result of copper uptake.

That Chlorella cells do indeed have a high affinity for heavy metals was stressed further by the work of JENNETT et al (1979). The authorsdiscussed the uptake of heavy metals by algae from effluents leaking from mining and milling waste. Us ing a so-called "algal meander system" in which contaminated water is caused to flow through long shallow channels,housing algal colonies, it was found that up to 99% of the heavy metals could be removed from solution. This example illustrates the use to which the high algal affinity for such pollutants can be put.

STEEMANN-NIELSEN and KAMP-NIELSEN (1970) looked at the deleterious effects of copper on the growth of Chlorella cultures. They said that several substances, e.g. iron and citric acid, could counteract the effects of copper, and that initial cell concentration is of great importance in governing the magnitude of the response to the toxin. "The effect of a certain copper concentration stops at a certain concentration of the algae regardless of whether the experiment is started at this cell concentration or this concentration is obtained during the experiment. This is due to the binding of copper by the organic matter of cell walls and slime envelopes".

The above authors also made the point that H^+ ions can compete with the copper both when linking to ligands of the cell walls and when combining at the active sites of the cell membrane. This, they say, explains why the influence of copper is only slight at pH 5, for example, compared with that at pH 8. They believed that the

algae are not necessarily killed by copper levels that completely inhibit growth as, when placed in fresh medium, copper-treated cells could recover. Despite all of these variables that were quoted as being of relevance in Chlorella toxicity studies, STEEMANN-NIELSEN and KAMP-NIELSEN (1970) produced a graph, reproduced below as Fig. 1.1, showing the depressant effect on algal growth of various levels of applied copper in a growth medium.

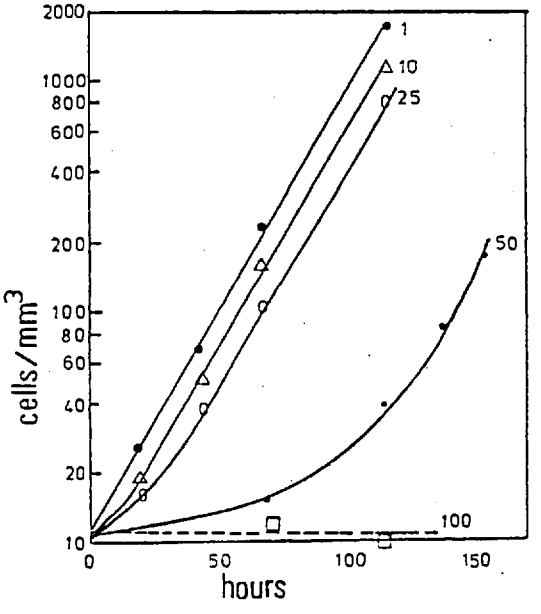


Fig. 1.1 Legend (after STEEMANN-NIELSEN and KAMP-NIELSEN, 1970): The growth of Chlorella (7 Klux, 20 C) as a function of time at varying concentrations of copper. Österlind "B" medium with 1/10 micro 1. The figures on the curves denote concentration of copper in µg/1.

Some interesting results were quoted, again with reference to Chlorella, by McBRIEN and HASSALL (1967). The paper reported severe growth inhibition, as well as of respiration and photosynthesis, when cells absorbed copper under anaerobic conditions, but that this did not occur when the metal was absorbed aerobically. If, after aerobic copper absorption, the cells are exposed to a period of anaerobiosis, then respiratory inhibition is as great as in the above case, but it usually gradually recovers as growth is not so drastically affected as it is with anaerobic copper uptake. The conclusion was that the extra-toxic copper, absorbed anaerobically, becomes bound to sites not normally available under aerobic conditions. FREY-WYSSLING (1953) has reported that anaerobiosis causes a decrease in protoplasm viscosity. He explained this by saying that, with no available oxygen to act as a hydrogen acceptor, metabolic reductants could reduce the disulphide linkage in protein to sulphydryl.

According to PASSOW et al (1961), of all the heavy metal toxicants, copper is the one to bind most strongly to sulphydryl groups. McBRIEN and HASSALL (1967) go on to say that "since disulphide linkages are of great importance in maintaining the tertiary and quarternary structures of proteins, the binding of copper to reduced disulphide linkages under anaerobic conditions could inhibit the restoration of the normal configuration of some metabolically-important proteins when aerobic conditions are resumed". They believed that it is unlikely that any one such effect could explain copper's anaerobic toxicity, although there is evidence that copper is the only heavy metal to act in this specific way.

The above explanation, if true, fits in well with HASSALL (1963) who said that, amongst metals, copper is unique in that it is "highly toxic when applied to (Chlorella) cells under anaerobic conditions, but seldom reduces respiration for many hours when applied at high concentrations in aerated vessels". He said that this difference cannot be explained by the ease of uptake in either the presence or absence of air, "although colour changes in poisoned cells may indicate different sub-cellular distributions".

KANAZAWA and KANAZAWA (1969), using Chlorella ellipsoidea, demonstrated that, with cupric ions present, cellular division in the dark is specifically inhibited - especially strongly at pH 6.3. They said that one possible explanation was that copper inhibited some biosynthetic process, preceding division, because the toxic effect was less pronounced under continuous light than in darkness, when the plant is more likely to be living off its reserves. They assumed that "since no cellular division took place in the presence of 3×10^{-6}M Cu^{2+}, despite the completion of nuclear division, it may be that "the process suppressed by Cu^{2+} is probably concerned with some events related to the cell membrane rather than with those occurring inside the cell". The suppression of cell division causes an accumulation of photosynthetic products and leads indirectly to its depression (STEEMANN-NIELSEN and KAMP-NIELSEN, 1970).

The above reference (KANAZAWA and KANAZAWA, 1969) that cell division was halted by copper even if nuclear division was complete was perhaps contradicted by a statement of STEEMANN-NIELSEN and KAMP-NIELSEN (1970) to the effect that, although the influence of copper depends on the divisional stage of the alga, "if the initial steps of cell division have taken place, the cell continues to divide".

However, this conclusion was based on work done with Chlorella Pyrenoidosa, and not C. ellipsoidea, and this may explain the difference.

ROSKO and RACHLIN (1977) also looked at copper's effects on Chlorella cell division. The authors reported that 0.18± .003ppm copper reduced cell division by 50%. Cells grown in 0.32 ppm copper were significantly larger than controls after 33 days and this level also caused an increase in the chlorophyll a content of the alga. They summarised by postulating that copper exerts a greater effect on cell division than on chlorophyll a content in C. vulgaris – and vice versa for zinc and lead.

That there can be considerable variation in the response of different plant species to copper's action is undisputed. GIBSON (1972) found that Anabaena flos-aquae was more sensitive to copper than was Scenedesmus quadricauda which also accumulated less of the metal. It was also stated that the sensitivity of the Anabaena culture declined as the culture aged. MALONEY and PALMER (1956) have looked at copper toxicity to thirty different species of algae and concluded that "copper sulphate appeared to be selective rather than general in its algicidal effects when used at concentrations of 2ppm or less".

ERICKSON (1970 and 1972) studied copper toxicity in the marine alga Thallassiosira pseudonana. The alga was sensitive to copper right down to 0.1μM where a reduction in growth rate was still observable. LEWEY (1977) looked at copper's effects on a brown, a green and a red marine alga (Sargassum muticum, Ulva spp. and Ceramium rubrum, respectively). Copper applications in the (relatively high) range of 10 – 50 ppm were lethal to all three species.

While investigating a potential aluminium requirement for citrus plants, LIEBIG et al (1942) found that some of the observations of this metal causing a slight growth stimulation might have been the result of an antagonistic effect of copper. They found that 0.1ppm copper was toxic to orange and lemon cuttings, while the addition of a further 0.1ppm aluminium was sufficient to offset the inhibition. If only a trace amount of copper was initially present, and then aluminium was added, a slight growth depression was noted. It may therefore be that the presence of aluminium in some way makes it more difficult for the plant to take up copper.

Another paper which looked at copper toxicity in relation to a second metal, this time silver, was that of YOUNG and LISK (1972). They found a synergistic effect of copper and silver ions on the growth of a range of green and blue – green algae. A similar investigation of metal interactions was conducted on barley by BECKETT and DAVIS (1978). It was found that the uptake of nickel and zinc into the shoots did not affect the amount of copper also being absorbed. They said that "the toxic effects of copper and zinc appear to be antagonistic when the tissue concentrations of both are above their critical levels" (when toxic effects become apparent). It may be that the two metals, once inside the plant, are competing for the same sites of action. VELTRUP (1979), however, reported that the uptake characteristics for copper into barley roots were altered in the presence of Ni^{2+}, Cd^{2+} or Co^{2+}.

In studies looking at the uptake of heavy metals into a variety of crop plants, BECKETT and DAVIS (1977) and DAVIS and BECKETT (1978) found considerable variation in the concentration of copper, nickel or zinc in the tissues of young barley plants grown in nutrient solution with metal additions, because of the various possible growing conditions. However, the minimum concentration of these metals in the plant tissues necessary to cause toxic reactions were found to be almost independent of the growing conditions. For copper, this critical level was quoted as being 19 ppm on a plant dry weight basis. These workers obtained similar copper concentration values to this for ryegrass S.23 (21 ppm), lettuce (21 ppm), rape (16 ppm) and wheat (18 ppm). These results of DAVIS and BECKETT seem to provide evidence that, whatever other factors are pertaining, as soon as a critical copper concentration is reached, toxic systems will develop.

BAZZAZ et al (1974) and LAMOREAUX and CHANEY (1978) looked at the uptake of cadmium into excised silver maple, corn and sunflower leaves. They demonstrated that the degree of uptake was dependent on the level of metal supplied and that the metal caused a reduction in net photosynthesis and transpiration of the plants. This effect, they attributed to the metal causing stomatal closure.

PUCKETT et al (1972) and PUCKETT (1976) monitored the uptake of heavy metals into lichens. In the earlier paper, they found that the relative capacities for uptake from solutions containing a single metal ion were :

Fe, Cu > Ni > Pb > Co > Zn. However, studies on competitive uptake with solution containing more than one metal revealed a selectivity sequence as follows: Fe $\gg$ Pb > Cu $\gg$ Ni, Zn > Co. The authors said that the sequences accorded with a cation uptake mechanism involving ion exchange modified by metal-complex formation. The 1976 paper said that copper, mercury and silver produced a gradual potassium loss from the thallus, while most other metals caused an abrupt efflux at a paticular metal dose level.

Clearly, from the above review on copper uptake and accumulation, one can appreciate the wide variation that exists in approaches to these studies. Specific reference has been made to copper in algal systems but, even so, a wide range of parameters have been monitored by authors employing on even wider range of subject species. Having looked generally at copper uptake into plants and particularly at its grosser effects on over all algal growth, it will now be of interest to consider its implications in a more specific field, its effects on photosynthesis.

1.6 Studies on the physiology of photosynthetic inhibition by copper:

Most of the work to look at copper's effects on photosynthetic processes has either used green algae or isolated chloroplasts as the experimental material. GREENFIELD (1942) looked at the inhibitory effects of a range of inorganic compounds on photosynthesis in Chlorella cells. He found that only very low concentrations (10^{-7}M) of copper sulphate were required to significantly inhibit photosynthesis, as measured by oxygen evolution. By means of studies at five different light intensities, copper sulphate was found to be mostly retarding the dark reactions of photosynthesis, with a lesser inhibition of the light stage. MACDOWALL (1948), however, looked at the effects of various inhibitors, including $CuSO_4$, on the photochemical reduction of a dye by isolated chloroplasts. His results indicated that copper was more damaging to the light phase of the process than to the dark, 10^{-5} M causing a 50% inhibition of photochemical indophenol reduction.

Since this early work, there have been many postulated explanations as to the mechanisms and sites of photosynthetic inhibition by copper. Rather than looking for one all important effect, it has probably been more useful to look at the relative sensitivities of the various processes damaged by the metal.

Further work on Chlorella was conducted by STEEMANN-NIELSEN et al (1969). Results were quoted to show that if a single salt solution of copper was used, then copper penetrates immediately into the plasma of the cells, causing a reduction in photosynthetic rate at both high and low light intensities. If, however, copper sulphate is added to culture medium (Österlund, pH 8), it takes some hours before any effect of a deleterious copper concentration is observed, and then initially only at light saturation. The work further showed that the influence of the copper increased with decreasing algal concentration and that if a culture medium at pH 5 was used instead of the normal one at pH 8, then copper concentrations about ten times as high must be employed in order to give the same deleterious effect. The paper concluded by saying that the effect of copper on Chlorella cells in a balanced medium "is not due to a marked penetration of this ion into the plasma but to a binding to the cytoplasmic membrane whereby the cells become more or less unable to divide. The cells become saturated with assimilation products which have a depressant effect on photosynthesis". Other cations, including H^+, seem to compete with copper for the active sites on the membranes. It must be said that it seems likely that the above scheme of events could only prove true where the level of applied copper was relatively low; a higher dose could presumably poison various internal enzyme systems before the build up of metabolites or assimilates could have an effect.

In further work, STEEMANN-NIELSEN and LAURSEN (1976) reduced the photosynthetic rate of phytoplankton living in water samples from four Danish lakes, bythe addition of copper. Among their findings were observations that, amongst the factors which influenced the toxic action of Cu^{2+}, were the taxonomic composition of the phytoplankton, their numbers, the humus content of the water and pH. These results are in keeping with previous findings by these and other authors.

GROSS et al (1970) found that the addition of copper to non-growing cells of a normal Chlorella culture caused a reduction in photosynthesis, measured polarographically, concurrent with a reduction in total pigments and a blue shift of chlorophyll absorption. CEDENO-MALDONADO and SWADER (1974) also obtained a reduction in cellular chlorophyll on adding copper and demonstrated that anaerobiosis and light treatment increased the effect. (see Fig. 1.2).

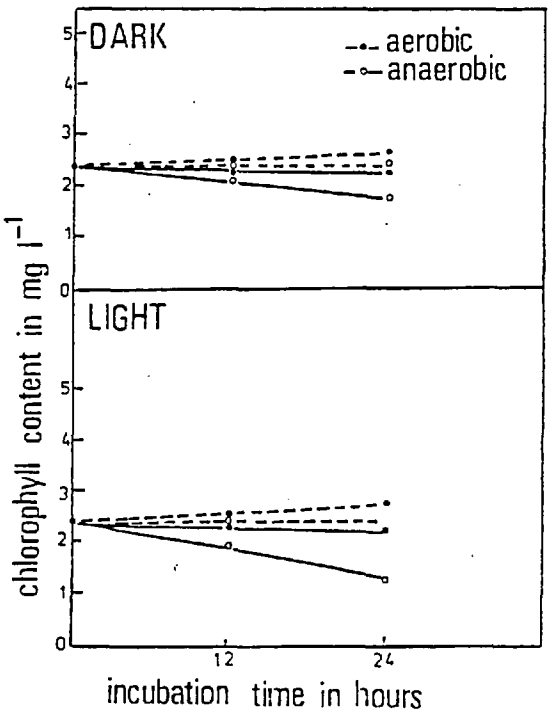


Fig. 1.2 Legend (after CEDENO-MALDONADO and SWADER, 1974). Effect of the cupric ion on the chlorophyll content of Chlorella. Broken lines represent the control and solid lines are results with 100 μM $CuSO_4$. The cupric ion was added to give an initial ratio of 1.27 ug Cu^{2+}/ 10^6 cells. Values are averages of four replications and the results of the cupric ion treatment in dark and light (23.7 Klux) under anaerobic conditions are significantly different at the 5% level of probability.

In these results, photosynthesis was inhibited within two minutes of adding 1mM cupric ions, but it was said that the percentage inhibition depended on the ratio of cells to copper present. Cells incubated for even just a short time in copper levels that completely inhibited photosynthesis were, if subsequently transfered to fresh medium, completely unable to recover. This was taken as an indication that the photosynthetic apparatus, and therefore the capability for autotrophic growth, had been destroyed.

STEEMANN-NIELSEN and KAMP-NIELSEN (1970) had found regrowth in these circumstances, but their medium had contained a chelator which was not present in CEDENO-MALDONADO's experiments. We have already noted how greatly the presence of chelator can effect copper toxicology.

DE FILIPPIS and PALLAGHY (1976) found that levels as low as 10 μM of mercuric chloride or 1 μM phenylmercuric acetate could cause significant difference in pigment levels in Chlorella cells after just a thirty minute dark preincubation period. They said that "one of the primary effects of heavy metals on photosynthesis is pigment destruction". PUCKETT (1976) stated that copper, mercury and silver all cause a displacement and a decrease in the absorption spectrum of chlorophyll from the lichen Umbilicaria muhlenbergii (obviously including an algal symbiont).

In CEDENO-MALDONADO's paper in 1974, it was demonstrated that photosynthesis was considerably more sensitive to copper inhibition than was respiration. (Fig. 1.3)

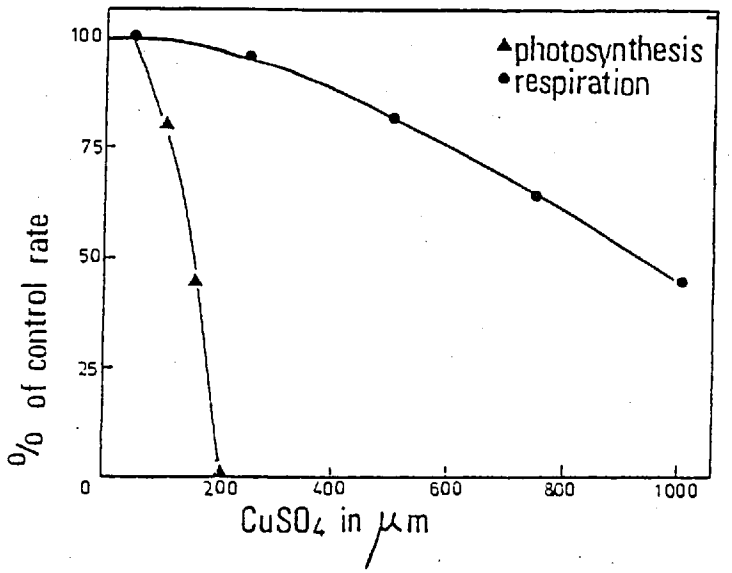


Fig. 1.3 Legend (after CEDENO-MALDONADO and SWADER, 1974). Effect of the cupric ion on the photosynthesis and respiration of Chlorella. Assay system (as described in his materials and methods section) with 8.5×10^6 cells containing 32 μg chlorophyll. Control rates were 240 μmoles O_2 evolved/hr./mg chlorophyll in the light and 55 μmoles taken up/hr./mg chlorophyll in the dark. The reactions were measured five minutes after adding the inhibitor.

The level of copper supplied also had a profound bearing on the time-course of the photosynthetic response to copper, as illustrated by another of the authors' graphs (Fig. 1.4)

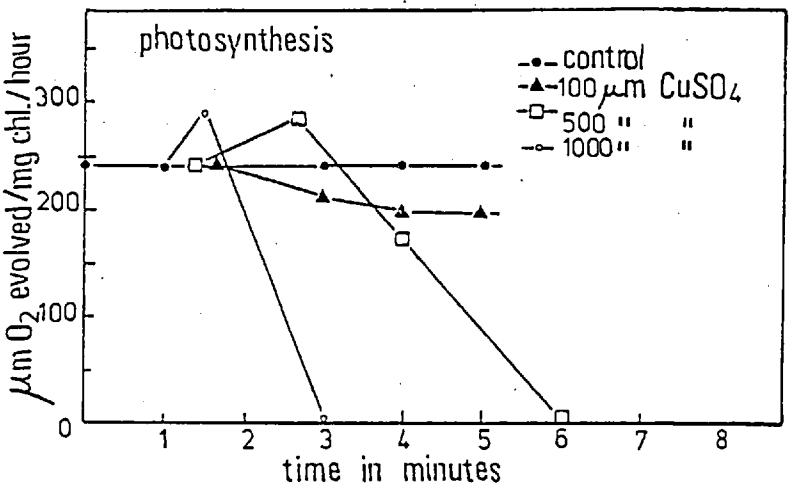


Fig. 1.4 Legend (after CEDENO-MALDONADO and SWADER, 1974). Time course of the cupric ion inhibition of photosynthesis in Chlorella. Assay system (as described in his materials and methods section) with cells containing 32 µg chlorophyll.

It was hypothesized that the initial rise in photosynthesis observed at the highest copper treatments may have been caused by the cupric ion inducing an increase in the permeability of the chloroplast membranes, "allowing an increase in the availability of substrates". The highest concentration of the metal used (1 mM $CuSO_4$) caused visible bleaching of the cells after fifteen minutes, regardless of whether incubation had been in the light or the dark.

The increase in membrane permeability, attendant upon a toxic metal treatment, was also mentioned by KAMP-NIELSEN (1971) who concluded that, although this leakage from the membrane, leading to an outflow of potassium ions, was the primary action of both mercury and copper poisoning, the leakage did not seem to be associated with the decrease of photosynthesis.

STEEMANN-NIELSEN and WIUM ANDERSEN (1971) demonstrated that ionic copper affected the photosynthesis rate of the diatom Nitzschia palea. A difference between this diatom and Chlorella was noted in that Nitzschia responded to a copper shock by excreting organic material. This material was able to bind to some of the copper in solution and therefore, having effectively detoxified a portion of the metal,

the diatom's growth could recover more quickly than could that of Chlorella. HOSTETTE (1973) used Chlamydomonas moewusii and Cyclotella meneghiniana in his work. Chlamydomonas proved to be relatively copper resistant, being unaffected by up to 2 mg copper per litre. Six mg of copper per litre reduced nett photosynthesis to 70% of controls, but with Cyclotella only 0.8 mg l^{-1} reduced the rate to 35% of the normal level.

In 1972, CEDENO-MALDONADO et al, working this time with isoloted spinach chloroplasts, had found a strong inhibition of uncoupled photosynthetic electron transport by Cu^{2+}, the inhibition being dependent on the chlorophyll:copper ratio. When the chloroplasts were subjected to preincubation in the light, there was increased inhibition as a result of copper binding to inhibitory sites, whereas pre-incubation in the dark seemed to result in binding to non-inhibitory sites and decreased inhibition. It was concluded that the degree of inhibition was lower at low light intensites than at high. They also assayed the two photosystems seperately and found PSII to be the more susceptible of the two to copper damage. (Fig. 1.5)

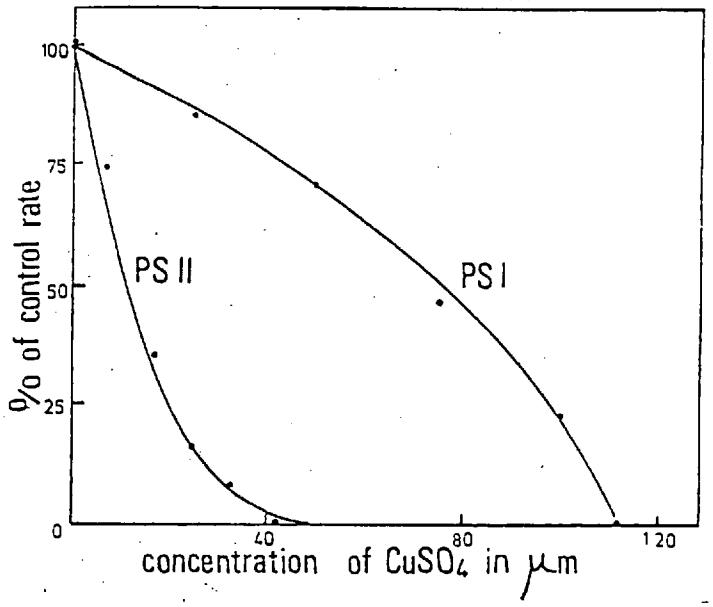


Fig. 1.5 Legend (after CEDENO-MALDONADO et al, 1972).
Effect of Cu^{2+} on uncoupled electron transport in photosystems I and II of isolated chloroplasts. PS II assay, reaction medium (3 ml) contained: 30 mM K_2HPO_4, pH 7.6, 0.25 M sucrose, 1mM $(NH_4)_2SO_4$, 0.67 mM 1,5-diphenylcarbazide, 0.33 mM DCIP, and chloroplasts (65 µg chlorophyll). Control rate was 108 µmoles DCIP reduced /mg chlorophyll /hr. PS I assay, reaction mixture (2 ml) contained:50 mM Hepes, 50 µM 2-anthraquinone sulphonic acid, 0.25 mM DCIP, 1mM ascorbate, 1mM $(NH_4)_2SO_4$, 2.5% ethanol, catalase (74,000 e.u.) and chloroplasts (61 µg chlorophyll). Control rate was 317 µeq of O_2 uptake /mg chlorophyll /hr.

OVERNELL (1975) worked with Chlamydomonas reinhardii and looked at inhibition of the Hill reaction (electron transport from water to dichlorophendindophenol – DCIP) by copper. He produced a curve depicting percentage inhibition of the control against copper concentration. The slope of the trace increased with increasing copper levels and he said that this was because of the "additive effects of the action of Cu^{2+} on two enzyme systems, both of which are required for activity". He hypothesized that one of these systems would bemore sensitive to copper, having a low number for n (the number of metal ions bound to the enzyme site), while the other would need more copper for significant inhibition. Thus, as more copper penetrated to these sites, either as a result of a longer period of exposure or because of a higher metal dose, the photosynthetic inhibition would, up to a point, become progressively greater.

SHIOI et al (1978a) looked at copper in relation to partial electron transport reactions in spinach chloroplasts. Two quite different inhibitory effects were found, one acting on PS I and one on PS II. Copper was found to inhibit ferredoxin-catalysed reactions such as $NADP^+$ photoreduction. The concentration required for 50% inhibition was about 2 μM copper sulphate. They found no effect of copper, however, if methyl-viologen was used as the final electron acceptor, electrons being donated by DCIP.

The second inhibitory site was found to exist somewhere at the beginning of PS II. This system, in effect, passes electrons, released after water-splitting, up a redox gradient to reduce a substance often termed "Q", the PS II light act providing the necessary energy to drive the process. In the experimental system, DCIP is used to accept electrons in the vicinity of Q and it was this overall reaction that was copper-sensitive. It was further found that diphenylcarbazide, (DPC), which can normally donate electrons to a site between the reaction centre of PS II (on which the light acts) and the water-splitting system (VERNON and SHAW, 1969), could not overcome the inhibition. This was therefore good evidence that copper was acting at a site after the point of electron donation byDPC, i.e. between the primary oxidising site of PS II and the electron donating site of DPC.

In further work, this time with broken cells of the green alga Ankistrodesmus falcatus, SHIOI et al (1978b) found that DCIP photoreduction was more sensitive to

copper inhibition in this alga than in spinach chloroplasts. Again, the lost activity of the system could not be restored by the use of the artificial electron donor DPC. Inactivation of the DCIP Hill reaction reached 45% after incubation with 10 μM cupric sulphate for 20 minutes.

The fluorescence activity of the cells, whereby the chlorophyll molecules emit light of a wavelength other than that of the incident light, is dependent on the reduction of substance Q (and therefore dependent on a flow of electrons from the PS II action centre to Q) (DUYSENS and SWEERS, 1963 and DUYSENS, 1963). The level of fluorescence was found to be reduced by copper additions (SHIOI et al, 1978b), DPC again having no restorative effect. The same conclusion as before was therefore arrived at, i.e. that copper was inhibitory at a site between the point of DPC electron donation and the oxidising side of the PS II reaction centre. Further evidence of the effects of light intensity seemed to point to the fact that copper mostly affected the reaction rate of the dark step and had less inhibitory effect on the quantum efficiency of the primary reaction of electron transport in PS II.

Further evidence for this sort of scenario in metal involvement in photosynthesis is given in the paper on the subject of the effects of cadmium on PS II in isolated chloroplasts by DUIJVENDIJK-MATTEOLI and DESMET (1975). They showed that cadmium too was an inhibitor of the electron transport chain on the water-splitting side of PS II. In this case, hydroxylamine which, like DPC, is an artificial donor supplying electrons before the PS II reaction centre, could completely bypass the cadmium effect, indicating that this metal was inhibitory at a site before the site of hydroxylamine donation.

Because the electron donors of PS II are localized either inside or on the inner surface of the thylakoid membranes of the grana (BLANKENSHIP and SAUER, 1974), the copper and cadmium, in order to exert an effect, have to penetrate the membranes. DUIJVENDIJK-MATTEOLI and DESMET (1975) showed that the cadmium inhibition of electron transport or the transport of cadmium through the membranes (or both) were dependent on the physico-chemical form of the applied cadmium. It was shown that the electron transport chain was more sensitive to Cd^{2+} than to complexed cadmium. This may be of relevance to the copper situation, remembering some of the studies on complex formation conducted with this metal.

MILES et al (1972) had demonstrated photosynthetic electron transport inhibition by lead in isolated chloroplasts and had also narrowed down the site of action to be on the oxidising side of PS II. Again, it was found that the use of hydoxylamine could restore normal fluorescence levels. HONEYCUTT and KROGMANN (1972) found that, although phenylmercuric acetate seemed to selectively inhibit a PS II site close to the Mn-mediated water-splitting event, and although DPC was ineffective as a donor to overcome the inhibition, the addition of either hydroxylamine or hydroquinone plus ascorbate (another artificial electron-donating system) was able to restore electron flow.

HONEYCUTT and KROGMANN (1972) presented a scheme to explain their finding and this is reproduced below as Fig. 1.6

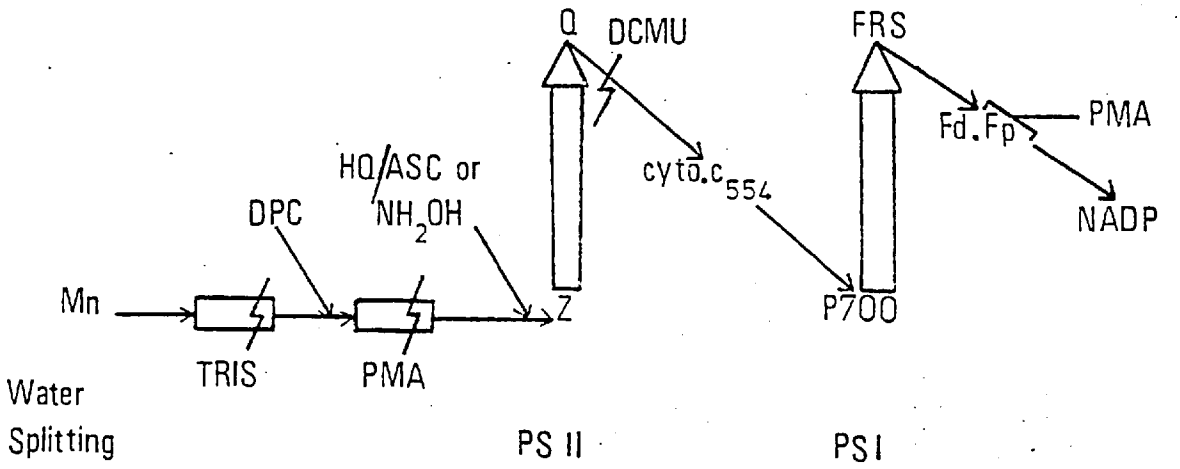


Fig. 1.6 Legend (after HONEYCUTT and KROGMANN, 1972).
Inhibition of photosynthetic electron transport. Tris buffer prevents the use of electrons from manganous ions (Mn) and this block is circumvented by diphenylcarbazide (DPC). High concentrations of phenylmercuric acetate (PMA) will block electron flow from both Mn and DPC, but can be bypassed by using either hydroquinone and ascorbate (HQ/Asc) or hydroxylamine (NH_2OH) as electron donors to the photoact ($Z \rightarrow Q$). All of these reactions are inhibited by DCMU. Low concentrations of PMA block the activity of ferredoxin (Fd) and the ferredoxin-NADP oxidoreductase (Fp) which prevents the flow of electrons from cytochrome C_{554} (Cyto. C_{554}) and other carriers in the intermediary dark electron transport chain through P700, PS I and the ferredoxin reducing substance (FRS) to NADP.

The relative points of DPC, NH_2OH and HQ/Asc. electron donation are not altogether clear and it is therefore not possible to say whether or not these observations point to different sites of action for the metals mentioned. This perhaps seems likely, but that the inhibitory sites are at least in the same area of the electron transport pathway does seem to have been clearly demonstrated.

HABERMANN (1969) investigated the reversal, partial or otherwise, of copper's inhibition of photosynthesis by manganese. Her results showed that manganese could reverse copper's inhibition of the "Mehler reaction" (a modified Hill reaction in which electron transport from water utilised molecular oxygen as the electron acceptor). This author also worked on the manganese-dependent photo-oxidation of diketogulonic acid which can be mediated by isolated chloroplasts. This oxidation could be poisoned by copper ions, but restored by the subsequent addition of manganese. In fact, in each of the chloroplast-mediated reactions that were studied, HABERMANN found that "manganous ions reduced sensitivity to copper inhibition".

The main explanation put forward for these results centres around the idea that the role of manganese in photosynthesis is concerned with an involvement in oxygen evolution (McKENNA and BISHOP, 1967), i.e. in a reaction closely associated with PS II. The antagonism between copper and manganese in several types of chloroplast reactions seems to indicate that copper affects the site of manganese action; if there were competitions between the two metals for positions at a particular enzyme site, and if the manganese-enzyme complex was active and the copper-enzyme system inactive, then this could explain the copper inhibition and the manganese-reversal.

A secondary, or complementary, explanation might take account of the effects of these ions on membrane permeability. HABERMANN does not definitely choose between these two explanations, but does make the statement that "added manganese has a protective effect against the generally inhibiting action of copper on the photosynthetic apparatus". It is perhaps some/what difficult to understand how Mn can cause a decrease in all of the adverse effects of copper on photosynthetic processes, particularly in the light of the above mentioned model for copper-involvement in the "Z scheme". Some of HABERMANN's work cannot therefore be fitted into an overall scenario, but it can help to illustrate the diversity of theories and the multiplicity of approaches to the work.

Investigations in this field of metal toxicology and plant photosynthesis have therefore tended to support the gradually established idea that the components on the oxidising side of PS II are the most sensitive to Cu^{2+} inhibition of photosynthetic electron transport. (CEDENO-MALDONADO and SWADER, 1974).

However, the variety of copper effects force us to add that metal addition does have other consequences for the photosynthetic tissue. Thus STEEMANN-NIELSEN et al (1969) were probably justified in saying that when copper really penetrates into the cells of Chlorella, there is a decrease in the rate of both the light and the dark reactions in photosynthesis.

However, a specific restorative effect of copper on the photosynthesis of tris-washed potato tuber chloroplasts was demonstrated by RAMASWAMY and MADHUSUDANAN NAIR (1978). They showed that the tris wash removed 15% of the copper normally associated with the chloroplasts and that only the addition of further copper (as "Cu-BSA complex" with bovine serum albumin) was effective in restoring photosynthesis levels. In the light of other work on copper's inhibition of photosynthesis, it is likely that this is a specific case or that the effective level of the metal was so low as to be non-inhibitory. The use of a Cu-BSA complex undoubtedly reduced copper's possible toxicity.

1.7 Copper interference in respiratory processes.

HASSALL (1962) demonstrated a specific effect of copper on the respiration of Chlorella vulgaris. The results showed that respiratory inhibition could be obtained with many metals, but that several hours often had to elapse before the effect was noticeable. Copper, however, was found to be an exception, the speed of its action apparently depending on the environmental conditions pertaining during the period of treatment. For example, it was found that if the suspension of Chlorella were shaken continuously (i.e. aerated), 10^{-1}M copper sulphate was not inhibitory for 7-20 hours, but if the shaking was stopped, concentrations lower than 10^{-3}M rapidly became toxic. Although analysis showed that there was greater uptake of copper by the unshaken cells, this was probably due to the relatively rapid death of these cells, as dead cells absorb copper more rapidly than do live ones. His figures showed that the fall in respiration in unshaken vessels was not closely related to the amount of applied copper or to the

amount present in the cells.

Lack of oxygen was therefore shown to be a factor leading to a high degree of copper inhibition of respiration; Chlorella cells shaken with copper, but in an atmosphere of hydrogen and not of air, suffered similar, seemingly-irreversible respiratory inhibition. HASSALL (1962) concluded that, although a change in the form of the copper applied under anaerobic conditions could not be completely discounted, there could perhaps be two more likely explanations. Possibly, internal distribution of copper in the absence of air is different from when air is present. This explanation discounts the fact that the eleven other metals also tested show no such dependence on environmental conditions for their effects to be manifest. His other idea was that, under anaerobiosis, an alternative respiratory pathway may exist for which copper was a highly-specific poison.

CEDENO-MALDONADO and SWADER (1974) also studied the action of copper on Chlorella respiration. The results showed respiratory inhibition by copper, but also demonstrated that photosynthesis was far more sensitive. Measurable inhibition of respiration required twice the amount of cupric ion as that required to give measurable inhibition of photosynthesis. Respiration was only slightly inhibited by concentrations of the cupric ion which were 100% inhibitory to photosynthesis.

HASSALL (1967) followed up previous work with a study involving a strain of Chlorella vulgaris in which respiration was insensitive to Fluoride ions and, in aerobic conditions, copper ions. However, even in the presence of air, when copper and fluoride ions were added simultaneously, oxygen uptake almost ceased immediately. No similar effect could be seen when other metals were substituted for copper or when most other anions were used to replace fluoride (the two exceptions being cyanide and iodide, but both of these interact with ionic copper, causing complications).

The insensitivity of respiration to fluoride has often been attributed to the presence of an alternative respiratory pathway, not dependent on enolase (WARBURTON et al, 1951, working with Pseudomonas aeruginosa and McNULTY and LORDS, 1960, with Chlorella pyrenoidosa). GIBBS (1954) and ROSS et al (1962) have tentatively characterized this alternative respiratory sequence as being the pentose-phosphate pathway. HASSALL therefore suggested that the fluoride was inhibiting the normal

respiratory pathway and that the copper was in fact, inhibiting the pentose-phosphate chain that would normally come into operation as a form of back-up system.

A further paper by HASSALL (1969) continued the theme of this idea. This time, it was pointed out that, if the cells were pretreated in copper sulphate and then sodium fluoride added, the inhibition of respiration became more severe as the pretreatment time was lengthened, (except at copper levels so high that inhibition is complete at all lengths of pretreatment). When, however, the pretreatment was with fluoride ions, the inhibition was less as the pretreatment time was lengthened, eventually disappearing when the time interval was ninety minutes or more. It was again concluded that copper was acting by inhibiting the alternative pathway and the author quoted as supporting, if somewhat-circumstantial, evidence the work of TEWFIK and STUMPF (1951). Using leaf preparations, they previously had observed an oxidative system that was unaffected by fluoride (and cyanide) ions, and yet was almost completely inhibited by copper.

Thus, whatever the direct relevance of the TEWFIK and STUMPF work, HASSALL seemed to be gradually extending his theory and building up evidence for it. However, SARGENT and TAYLOR (1971) published some results that, while agreeing in part, also disagree with aspects of HASSALL's work. These authors confirmed that fluoride ions do cause inhibition of the enolase enzyme of glycolysis, but they also said that the second aspect of HASSALL's model, the complete inhibition of the alternative pathway by Cu^{2+}, was wrong and that, in fact, both Cu^{2+} and F^- ions are required to inhibit it entirely. In the opinion of these authors, HASSALL's observation that respiratory inhibition by copper decreased as the time of preincubation with fluoride increased was probably explained by the fact that fluoride is somehow removed or inactivated during the preincubation period and that, when the copper is added, glycolysis is in fact no longer being so effectively inhibited as was formerly the case.

1.8 Background to the approach adopted in the thesis.

The purpose of the work presented in this thesis was to explore further the action of copper on plant and algal growth and cellular biochemistry. Thus, the experiments attempted to follow a sequence whereby one gradually focussed-in on more and more specific copper effects.

Chlorella pyrenoidosa, a member of the chlorophyceae, was chosen as the main experimental material because it lends itself very well to growth and ion uptake studies. Interest in the species already exists in the literature and its unicellular nature renders it a relatively simple plant system. This is of particular importance in work involving a metal like copper as its near-universal reactivity with biological ligands means that, for complete understanding, one must concentrate on investigations into its primary effects. The use of Chlorella cells makes this considerably more possible than would be the case with higher plants where problems of metal binding at the root surface, slow and complex internal transport mechanisms and potential exclusion from certain parts of the plant might arise.

Some work was also conducted with the marine alga Sargassum muticum. Although not studied intensively, this species also proved to be a good experimental organism, and its use enabled some parallels to be drawn between the simple unicellular and larger, more complex algal systems.

In a later phase of the project the use of chlorophasts isolated from pea plants enabled copper's effects on photosynthesis to be more closely examined. The use of higher plant chloroplasts was adopted because it has been reported (CEDENO-MALDONADO and SWADER, 1974) that it is not possible to extract totally active chloroplasts from Chlorella cells. By isolating these organelles, one is further simplifying the system under scrutiny and increasing the chances of observing primary effects.

The experimental work of the thesis therefore fell into two broad categories:

1. Growth inhibition and ion uptake studies.

This approach, conducted with both Chlorella and Sargassum and using copper supplied as the sulphate or as a release glass, attempted to give a broad view of copper's effects on growth generally with a view to understanding dose-time relationships. The extent of any growth supression, the amount of metal accumulation by the tissues and the relevance of the activity of the cupric ion in relation to media composition were all investigated. These experiments were followed by further studies which looked at other, more specific metal effects.

2. Effects on cellular physiology, particularly at the level of photosynthetic electron flow.

This work, mainly using isolated chloroplasts, was further sub-divided into copper's interference with the overall electron transport chain and with the partial reactions associated with the two photosystems assayed seperately. The effects of various metal levels on both coupled and uncoupled rates of electron transport were assessed and the relevance, if any, of light or dark preincubation investigated.

Techniques of chlorophyll fluorescence measurement were also used in studies on photosystem II and various artificial electron donors employed in an attempt to pinpoint copper's inhibitory site.

CHAPTER II

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Work with Chlorella pyrenoidosa.

2.1.1 Maintenance of stock Chlorella cultures.

Reserve Chlorella cultures on standard agar slopes were kept in a cold-room at 4°C, but an actively-growing stock culture was maintained and used for the inoculation of the various growth and copper uptake experiments. The Chlorella stock originated from the Indiana culture collection and had previously been used extensively by BARBER (1967) and LUTON (1980).

The algal stock was cultured in a medium having the following composition modified after HEWITT (1964).

Chemical	Mol. wt.	$g\ l^{-1}$ stock sol^n	mls stock sol^n/ 2 litres final medium	ppm in final medium
1M KNO_3	101.10	39.102 g K	10	195.50 (K)
1M K_2HPO_4	174.18	78.204 g K	1	39.10 (K)
1M $MgSO_4.7H_2O$	246.48	24.312 g Mg	4	48.64 (Mg)
1M KH_2PO_4	136.09	39.102 g K	1	19.55 (K)
1M $Ca(NO_3)_2 4H_2O$	236.15	40.080 g Ca	0.5	10.02 (Ca)

Plus HUTNER's micronutrients (stock solution made up as below):

Element	At.Wt.	Supplied as	(Mol.Wt.)	g/500 mls stock sol^n	mg element in stock sol^n	ppm in final medium
Zinc	63.58	$ZnSO_4.7H_2O$	287.54	11.00	2501.1	10.00
Boron	10.82	H_3BO_3	61.82	5.70	997.6	3.99
Manganese	54.94	$MnSO_4.H_2O$	223.06	2.20	541.9	2.17
Iron	55.85	$FeSO_4.7H_2O$	278.02	2.50	502.2	2.01
Cobalt	58.94	$Co(CH_3COO)_2.4H_2O$	249.09	0.84	198.8	0.80
Copper	63.54	$CuSO_4.5H_2O$	249.69	0.78	198.5	0.79
Molybdenum	95.95	$(NH_4)_6Mo_7O_{24}$	1235.95	0.55	298.9	1.20
Ethylenediamine-tetracetic acid (EDTA)			(372.24)	(25.00)		(0.269 mM)

Two mls of HUTNER's solution were used per litre of final medium. 1 ppm is equivalent to 1 $\mu g\ ml^{-1}$.

The medium was autoclaved for 15 minutes at 120°C and a pressure of 30 lb. in^{-2}. Stock cultures were kept in vigorously-aerated dreschel bottles, the bubbling being sufficient to keep the cells in suspension. The bottles, kept at room temperature, with constant illumination provided by a 60 watt tungsten filament bulb, were frequently sub-cultured to keep the cells in the logarithmic growing phase. A thick dark green suspension could be obtained under these conditions from a very dilute initial inoculum in about four days.

2.2 Chlorella growth experiments.

2.2.1 Conditions employed in growth experiments.

The various growth experiments were conducted using a Gallenkamp orbital incubator which enabled up to 32 culture vessels (250 ml conical flasks), each containing 200 mls of medium, to be kept continously shaken at 22°C ± 2°C. In most experiments, four replicates were used per treatment and the siting of culture vessels on the shaker head was random. In initial experiments, an incubator with a timed fluorescent lighting facility was employed, supplying an average 67 μEinsteins M^{-2} $sec.^{-1}$ of photosynthetically-active radiation, and a 16 hour light, 8 hour dark photoperiod was used. Later, a second incubator without the built-in lights replaced the first and cells had to be cultured under continuous illumination. Aeration of the cultures was achieved by using a compressor (Edwards High Vacuum, model ECB1) which supplied ambient air, via a cotton-wool filter and two seperate water towers, to a plastic manifold, one arm of which, terminating in an Eppendorf syringe tip, delivered a fine stream of bubbles to each vessel. The use of clips to partially block the air flow to certain parts of the manifold near to the pump ensured equal aeration of all cultures. The medium destined for use as a particular treatment was made up in bulk and only then decanted into the various culture vessels, thus ensuring homogeneity of replicates.

2.2.2 Inoculation and monitoring of culture growth.

In most experiments, the cell inoculum was such as to give an initial packed cell volume (pcv) of 0.05%. This is a very dilute suspension. The pcv is a means of expressing cell density in a culture by reference to the proportion of the total available space that the cells actually occupy. Inoculation was therefore accomplished by using

moderate centrifugation (about 4000 rpm) to concentrate the stock Chlorella cultures in a smallish volume (up to about 50 mls) of fresh medium. The pcv of this new highly-concentrated stock was then found by injecting 1 ml of it into each of two graduated glass haematocrit tubes. These were spun at full speed (dial setting of 10) for standard ten minutes in an MSE bench centrifuge. An average pcv could then be determined for the stock and an appropriate volume of it be subsequently used to inoculate experimental cultures. Letting the pcv of the concentrated Chlorella stock = x %, and the required initial pcv for the cultures = y % (usually 0.05%), then the volume of stock needed in 200 mls is given by : 200 y/x (mls). The moment of cell inculation was taken as time zero.

In some experiments, particularly in work to look at copper uptake into cells, initial inocula of higher than 0.05% were used in order to try and bulk up samples for subsequent weighing.

After inoculation, the various cultures were allowed to shake for 10 or 15 minutes in the incubator before a 5 ml aliquot was removed from each vessel to act as an initial sample. The concentration of cells present was determined by reading the optical density (O.D.) of the suspension using a Unicam SP500 spectrophotometer. The O.D. was measured at 530 nm (arbitrarily chosen as being away from the main chlorophyll absorption peaks) against a sample of fresh medium acting as the blank. Aliquots were subsequently taken at intervals, enabling the culture development to be monitored over time. An initial experiment had revealed that increases in cell pcv up to about 0.45% (at least) had a linear correlation with the measured O. D. (about 2.0 for a pcv of 0.45%) of a sample aliquot. Therefore, cell O.D. readings were taken in the range of 0 to 2.00; this meant that some samples had to be diluted with fresh medium to bring them within the range, the dilution being subsequently allowed for.

2.2.3 Copper additions and proce dures adopted for the monitoring of copper levels in total samples, nutrient media and Chlorella cells.

In all of the experiments using a copper treatment, the metal was either added as the sulphate ($CuSO_4.5H_2O$, Analar grade, as supplied by B.D.H. chemicals ltd., Poole, England) or as a Control Slow Release Glass (RG). The sulphate was added as a small volume of a standard 100 or 1000 ppm (µg metal per ml) solution. Release Glasses

(B240177, of particle size >500 and < 710 μm, as supplied by Standard Telecommunication Laboratories Ltd. (STL), Harlow, Essex) are slow-dissolving compounds which gradually release metal to the medium and were added to cultures in small, weighed amounts.

In many experiments, an initial 5 ml sample of medium was removed from each treatment just prior to the inoculation with cells. Often, samples were taken at intervals during the experimental period, the cells, and RG if present, being removed by high-speed centrifugation. These samples, sealed in small polythene tubes and then stored, frozen solid, until they could be analysed, enabled a check to be kept on medium copper levels.

The samples were analysed at STL Ltd. using an Instrumentation Laboratory 151 atomic absorption spectrophotometer which operates using the principle that each element will absorb radiation of a particular wavelength, this being supplied by a specific lamp. The sample is aspirated into a high temperature oxygen/acetylene flame, positioned between the lamp and a detector; any copper in the sample is ionised by the high temperature conditions and will then absorb some of the incident radiation coming from the copper lamp. The amount of radiation reaching the detector is therefore inversely proportional to the amount of metal present in the sample. Calibration of the digital readout was effected using distilled water and a 1 ppm copper standard; all copper concentrations were read over this range in which the machine's response was linear, samples being diluted and the appropriate dilution factor being subsequently allowed for.

Where the monitoring of total copper levels (in the medium plus the cells) was undertaken, a known volume of culture was taken and dried down in a boiling tube in a drying oven. The resulting deposition was then digested by heating with 5 mls of an 80% nitric and 20% perchloric acid mixture which was boiled down to small volume (more acid could be addedif necessary) and then made up to 10 mls with distilled water. Analysis of this sample then enabled total copper levels in the initial sample to be calculated.

A similar proce dure was followed when Chlorella cells were digested, but here the alga was seperated from the medium by centrifugation and then resuspended in 4 mls of distilled water in an attempt to remove metal still associated with, but not bound by,

the cells. After centrifugation, the water was discarded and the cells, after recentifugation in another aliquot of distilled water were placed in preweighed tubes and dried down. Subsequent reweighing of the tubes gave the dry weight of the cells by difference and they were then digested and analysed as above. Results were usually quoted in µg of copper per gm of algal material (fresh weight basis). A factor was used to convert the dry to fresh weights. This was calculated by comparing the fresh weights of replicated fresh Chlorella pellets with the values obtained after the cells had been taken to constant weight in a drying oven.

In order to check the digestion technique, the same analysis procedure was used for several samples of "Bowen's kale", a standard biological material for which the elemental composition is well documented (Bowen, 1974).

2.2.4 Types of growth experiment undertaken with Chlorella.

Experiments were conducted to look at copper's effects on Chlorella growth with different conditions prevalent in the nutrient medium. The effects of a wide range of copper concentrations were assessed and further work investigated copper toxicity in media in which the presence of various chelator (EDTA) levels enabled the complexing capability to be changed. In some experiments, the micronutrient presence was varied, while other studies looked at the importance of time of exposure to the metal.

2.2.4.1 The use of a Control Slow Release Glass in Chlorella experiments.

The slow release glass B240177 was used to investigate copper's effectivity as a growth inhibitor in Chlorella when the metal was supplied in a form other than as the sulphate. Initially, some of the physical characteristics of the metal release were investigated to gain an idea of the copper levels appropriate for use with algae. Monitoring of copper release from the "glass" into several different aqueous samples was therefore undertaken. The appropriate medium was placed in a large vessel with constant stirring and RG added at time zero. Samples were then withdrawn, centrifuged and analysed over time. Release into distilled water, full Chlorella medium and filtered sea water was followed. In further experiments, copper release into media with and without micronutrients and chelator additions was monitored and work also looked at the build-up of levels when different cell inocula were present.

Normal Chlorella growth experiments were conducted using RG as the metal source. Cell growth, medium copper concentrations and metal content of the cells at the end of the experiment were all assessed. Various RG doses were employed and the experimental conditions were varied in order to try and obtain a graded growth response of the cells to increasing RG-mediated metal levels. In all growth experiments using these glasses a flask containing RG was included but not inoculated with cells; its O.D. was monitored with the other samples and any changes allowed for in the culture growth data. Further studies again investigated the relevance of the level of chelator presence to the growth inhibition caused by the glasses.

2.3 Copper uptake experiments involving Chlorella.

2.3.1 Indirect monitoring of copper uptake by Chlorella.

Changes had often been noted in medium copper levels after inoculation with cells. It was also known that Chlorella can affect medium pH values, and this itself could have effects on precipitation and the retention of metal in solution. Studies were therefore conducted to (i) characterise the nature of the pH effects and (ii) indirectly follow the cellular uptake by monitoring changes in metal levels in the bulk medium, allowance having been made for the pH changes.

A Pye Unicam model 290 pH meter was therefore used to follow the pH variations in illuminated and stirred Chlorella suspensions and, using either acid (HCl) or alkali (NaOH), similar changes were engineered in identical but cell-free vessels. pH effects were therefore allowed for and any difference noticed in the copper levels of medium aliquots from the vessels would be due to metal uptake or binding by the cells. Experiments of this type were conducted using various copper treatments and with different levels of EDTA addition in the medium.

2.3.2 Direct monitoring of copper levels in Chlorella and comparisons of metal uptake by live and scalded cells.

Studies involving a more direct following of metal uptake into Chlorella cells were conducted under differing experimental conditions. Chlorella suspensions, containing various copper levels supplied as the sulphate or as a RG, were sampled

over time and the metal contents of the cells assessed. The algal samples were digested as outlined above. Total and medium copper levels were also monitored so that an overall picture of changes in the distribution of metal within the system with time could be followed.

To investigate the possibility that dead tissues can still act as a sink for metals, comparisions were made of copper uptake into normal and scalded (i.e. dead) cells. The latter were prepared by suspending them in a test-tube in a water bath at 90°C for 5 minutes. Studies looked at uptake with different cell concentrations present.

2.4 Oxygen electrode work with Chlorella.

A Rank oxygen electrode (as supplied by Rank Bros., Bottisham, Cambridgeshire) was used to monitor the effects of various copper concentrations on photosynthesis and respiration in Chlorella cells. The apparatus consists of a central platinum cathode surrounded by a circular silver anode, and seperated from it by a saturated KCl solution. When a potentiating voltage of 600–700 mV is applied across the electrodes, a small current will flow, its magnitude being proportional to the oxygen concentration in the vicinity of the platinum cathode. This is probably because, under these conditions, the reaction

$$4e^- + 4H^+ + O_2 \longrightarrow 2H_2O$$

proceeds more readily than does

$$4e^- + 4H^+ \longrightarrow 2H_2$$

which normally occurs at the cathode during the electrolysis of water. The experimental solutions, containing algae or, in later studies, isolated chloroplasts, were contained in a 4 ml clear perspex reaction cell seperated from the electrodes by a teflon membrane permeable to oxygen. Changes in the oxygen concentration of the reaction medium were therefore monitored by reference to the changes produced in the cell below; for convenience, this current is measured as the voltage across a variable resistor. This is amplified and was displayed as a trace on a Rikadenki variable-speed chart recorder. Calibration was achieved by using O_2- saturated distilled water (0.25 μM O_2/ml) for the full-scale reading and dithionite solutions for the zero deflection. The electrode cell was illuminated by a Rank Aldis slide projector fitted with a red filter (giving a light intensity of 600 Watts metre^{-2} at the surface of the reaction vessel), and was maintained at 22°C using a water jacket fed from a water bath.

2.4.1 Experimental procedure employed in oxygen electrode work.

The algal cells used came from a standard suspension in full medium with a pcv of 1%. The following procedure was adopted:

(i) the cells were seperated from 4 mls of standard by moderate centrifugation (3000 r.p.m.) and were then resuspended in 4 mls of bicarbonate buffer (after CEDENO-MALDONADO and SWADER, 1974). This buffer was a 2 mM solution of 65% $NaHCO_3$ and 35% $KHCO_3$.

(ii) The cell suspension was placed in the electrode and normal photosynthesis allowed to operate for four minutes. The samples were kept well-agitated using a small magnetic stirrer.

(iii) An appropriate small volume of 100 ppm copper standard was added to the suspension via the capillary opening at the top of the reaction cell, and photosynthesis allowed to continue for a further six minutes.

(iv) The projector lamp was turned off, the reaction cell darkened and a trace for dark respiration obtained.

Results were quoted in μM O_2 evolution or uptake per minute for photosynthesis and respiration respectively. Some Chlorella samples, on removal from the oxygen electrode, were washed with 1 mM EDTA and then used to inoculate aliquots of fresh medium. Their subsequent growth performance was then monitored to see to what extent this had been modified by the short-duration metal treatment.

2.5 Copper's effects on chlorophyll levels and a:b ratios in Chlorella cells.

2.5.1 Experimental procedure employed in work with Chlorella pigments.

The effects on Chlorella pigment levels of aerobic and anaerobic conditions acting in tandem with a copper treatment were investigated. Anaerobic conditions are reported to exaggerate copper's effects (Hassall, 1962). Replicated samples were removed at intervals from various Chlorella suspensions and the cells, after being seperated out by centrifugation, were resuspended in 80% acetone, maintained in a water bath at 50°C. The cells were left to extract for 15 minutes and were then removed from the mixture by spinning. The volume of the extract was measured and total chlorophyll and chlorophyll a and b determinations were made after the method of ARNON (1948). Absorption readings for the samples were made using a Perkin Elmer

554 spectrophotometer, and the equations used in the calculations were as follows:

$$\text{Total chlorophyll} = \text{chlorophyll } \underline{a}\ (C_a) + \text{chlorophyll } \underline{b}\ (C_b)$$

$$= 0.0202\ D_{645} + 0.00802\ D_{663} \quad g\ l^{-1}$$

$$C_a = 0.0127\ D_{663} - 0.00269\ D_{645} \quad g\ l^{-1}$$

$$C_b = 0.0229\ D_{645} - 0.00468\ D_{663} \quad g\ l^{-1}$$

where D is the absorption reading at the given wavelength (nm).

Total chlorophyll levels were expressed as mg of pigments per gm of cell dry weight. The cell weights were obtained by taking measured volumes of cell suspension and drying them down in preweighed tubes. Subsequent reweighing gave the required data by difference. The effects of a range of copper concentrations as well as different initial cell inoculation levels, were investigated.

2.6 Work with the phaeophyte Sargassum muticum.

This marine alga has been inadvertently introduced into the Solent area of the south coast, and has had great success in colonising large areas of shoreline and in supplanting indigenous species. The plant's profuse lateral tips provide good standard replicated segments for growth experiments. Interest in the plant already exists in the literature (JONES and FARNHAM, 1973: FLETCHER and FLETCHER, 1975 a and b : GRAY and JONES, 1977: LEWEY, 1977).

2.6.1 Sources of Sargassum material and conditions used for its growth.

Healthy-looking Sargassum plants were collected fresh from the sea at two different locations in the Portsmouth area; one site was the moat at Southsea and the other was a jetty in Langstone harbour. The plants could be kept healthy for up to about two months by maintaining them in vigorously-aerated filtered sea water in a growth room at 16°C with a 16 hours on, 8 hours off photoperiod.

2.6.2 Growth experiments conducted using Sargassum muticum.

An initial experiment having shown that 5 cm. lateral segments grew considerably faster with 16 hours daily light than with 8 hours, this former photoperiod was always

subsequently used.

In growth experiments, 10x1 cm lateral tips were placed into each of four replicate 100 ml volumes of filtered sea water to which various copper additions had already been made. The length of the tips was assessed at intervals and the monitoring of copper levels in sea water was undertaken (a copper standard made up in sea water was used for the calibration of the atomic absorption spectrophotometer). The sea water was changed at regular intervals. At the end of the experiment, the tissues were digested to compare their various metal contents, the process used being indentical to that outlined above for Chlorella cells. The tissues were briefly rinsed in distilled water prior to acid-digestion.

2.6.3 Sargassum survival experiments using respiration as a criterion.

An experiment was established, as above, but using various RG additions as the copper source. Copper levels were again monitored and the tissues were also ultimately digested, but the metal effects were assessed, this time, by noting the percentage of segments still respiring at the end of a four day period of treatment. Respiratory activity was demonstrated using the reagent triphenyl tetrazolium chloride (TTC) which colours respiring tissue red. The red pigment produced is formazan, the result of a reaction between the tetrazolium molecules and hydrogen atoms released by the dehydrogenase enzymes, involved with the respiratory processes (MOORE, 1972). The replicate segments from one flask were transferred to a petri-dish containing 8 mls of a 1% (weight/volume) solution of TTC in sea water, left for two hours and then a count was made of the red-coloured tissues. A value could be calculated for the copper dose required to kill 50% of the plants over the exposure time (LD_{50}). This method for assessing percentage survival was also used in parallel investigations in which sulphate was used as the metal source.

2.7 Work with isolated pea chloroplasts.

The effect of copper-poisoning on overall electron transfer, covering virtually the whole system from the water splitting event to the final generation of reducing potential, was studied and the two photosystems assayed seperately for copper sensitivity. Work to look at PS II fluorescence was conducted in order to try and narrow down copper's point of influence still further .

2.7.1 Isolation of broken chloroplasts.

Pea seeds (Pisum sativum, "Feltham First", as supplied by Dobie, Samuel and Son Ltd., Llangollen) were soaked in water for several hours and then densely sown in vermiculite in seed trays, a layer of about half an inch of vermiculite being used to cover the seeds. The trays were kept watered in a heated green house with a 12 hour light, 12 hour dark photoperiod and, after about 8 or 9 days, the pea seedlings were 2 or 3 inches tall, at which point they were used for chloroplast extraction.

A grinding medium with the following composition was employed (after NAKATANI and BARBER, 1977) :

Chemical	gm/300 mls dist. H_2O	final conc.
Sorbitol	18.21	0.33 M
Mes (2(N-morpholino)ethane sulphonic acid)	1.17	20 mM
$MgCl_2$	0.60 ml of 1 M sol^n	0.2 mM

The pH of this medium was adjusted to 6.5 using 1 M tris (Tris (hydroxymethyl) amino-methane). This grinding buffer was cooled to an icy slurry and then fresh pea leaves were added to it in a cooled grinding container, 35 gm of plant material being used for every 100 mls of medium. A polytron (type PT 35 O.D.) with the standard attachment (PT 35/2 O.D.) and a speed setting of 5.5 was then used to thoroughly macerate the leaves, the grinding being done in a series of five second bursts.

The macerated pea slurry was filtered through 10 layers of butter muslin with a thin layer of cotton wool between the top two pieces. The muslin was squeezed so as to force most of the liquid through into a beaker on ice. The resulting filtrate was then centrifuged for three minutes at 6000 r.p.m., using a cooled centrifuge head. The supernatant was subsequently poured off and the chloroplast pellets resuspended in a low salt buffer (LSB). The chloroplasts were kept on ice throughout. The LSB was made up as follows (double strength) :

Chemical	g/250 mls dist. H_2O	conc. after final dilution
Sorbitol	30.0	0.33 M
Hepes (N-2-hydroxyl-piperazine-N'-2-ethane sulphonic acid)	1.2	10 mM

The pH was taken to 7.6 with 1 M tris base.

In fact, the chloroplasts were normally added to a small volume of cooled distilled water and then an equal volume of double strength LSB was added; the distilled water gives the organelles an osmotic shock and ensures that they are broken. This breaking of the membranes means that more of the sites, notably those on the inner surfaces, become exposed and can therefore more easily take part in biochemical reactions.

Most experiments conducted using these broken chloroplasts used a standard chlorophyll concentration in the reaction medium of 50 µg/ml. The chlorophyll concentration in the standard was calculated by a method modified after ARNON (1948). This entailed diluting a small volume (few µls) of chloroplast standard with 80% acetone, allowing the chlorophyll to extract for a minute or two and by then using a spectrophotometer to read the absorbance value at 652 nm. Then :

$$\frac{\text{Abs (652)} \times \text{dilution factor}}{36} = \text{chlorophyll concentration in mg/ml.}$$

LSB was used as the basic reaction medium in all electron transport studies. Additions of other reactants were added as appropriate.

2.7.2 Techniques used for assessing overall photosynthetic electron transport to methyl viologen.

Copper effects on photosynthetic electron transport through virtually the whole system to methyl viologen were investigated. The sensitivity of the isolated chloroplasts to various levels of copper addition was assessed by monitoring oxygen uptake from solution using a Rank oxygen electrode set up as before. The reaction cell was maintained at 22°C with constant stirring and bright illumination provided by a Rank Aldis slide projector positioned close to the electrode cell.

The standard reaction medium was typically composed of :

2 mls LSB (single strength)
40 µl 1mM MV (to give 20 µM)
100 µl 20mM azide (NaN_3) (to give 1 mM)
chloroplast standard (to give 50 µg chlorophyll 1ml)
copper additions (using 100 or 1000 ppm standards)

Azide was included to inhibit catalase activity. The normal proceedure was to add all of the reactants except methyl viologen to the reaction cell and to allow one minute in the dark for temperature equilibration. The light was then turned on and MV added at time zero. Two minutes of coupled electron transport were allowed and then 20 µl of 500 mM ammonium chloride, NH_4Cl, were added (giving a final level of 5 mM) as an uncoupler of electron transport, stopping phosphorylation (ATP production) and thereby removing the slowing-down effect that this process has on electron flow rates. The uncoupled rate was also assessed over a two minute period. All rates were expressed as µmole O_2 uptake/mg chlorophyll/hour; often, they were expressed as a percentage of the appropriate control rate.

2.7.2.1 Types of experiment used to assess copper's effects on overall electron transport to MV.

Chloroplasts, once isolated, had to be used within two to three hours as, thereafter, their activity declined. Experiments looked at the effects of various copper levels on both coupled and uncoupled electron transport, metal being added directly to the reaction cell. In other studies, the chloroplasts were preincubated in a copper-containing reaction medium on ice and sub-samples were taken over time to investigate any possible gradual development of toxicological effects. Experiments also compared the results obtained when preincubation was in the light and the dark. In these experiments, the MV was only added to a particular sample at the last moment, i.e. when it was placed in the reaction cell.

Studies were conducted to demonstrate that it was the cupric ion and not SO_4^{2-} that was causing the effects observed. Experiments therefore used a range of K_2SO_4 concentrations and also investigated how a chloroplast sample reacted to various times of exposure to a SO_4^{2-} concentration even higher than that found in most of the experiments involving copper.

2.7.3 Photosystem I assessment techniques.

To assess PS I alone, a partial electron transport system involving dichlorophenol indophenol (DPIP) at 0.1 mM plus ascorbate (Asc.) at 1 mM as the electron donating combination and methyl viologen at 20 µM as the acceptor was used. DCMU (3-(3',4'-dichlorophenyl)-1,1-dimethyl urea) was present at a level of 5 µM to block the flow of

electrons from PS II.

In order to overcome a complication whereby it was found that copper was acting as a catalyst and drastically increasing the uptake of oxygen by ascorbate (in agreement with GURD and WILCOX, 1956), a system was adopted which effectively kept these two reactants apart. Basically, therefore, the chloroplasts were preincubated on ice in LSB containing various copper treatments. Samples were removed at intervals and the organelles seperated out from the bulk solution using a one minute spin at 5000 rpm. The chloroplasts were then resuspended in fresh LSB, placed in the electrode, the other reactants added (in the order DCMU, DPIP, MV and Asc.) and the rate of oxygen uptake measured as usual. The time of ascorbate addition was taken as the marker for the time of exposure to the metal treatment.

2.7.3.1 Types of experiment conducted to assess copper's effects on PS I activity.

Various copper levels were used in studies to follow any possible graduul development of PS I inhibition. Experiments were also conducted to compare the effects of a particular copper level (5 ppm, 79 μM) on PS I - mediated oxygen uptake when chloroplasts were present at different concentrations. Chlorophyll levels were, however, not allowed to exceed 100 μg/ml. Both coupled and uncoupled transport rates were assessed.

2.7.4 Photosystem II assessment techniques.

In this work, the electron acceptor used was benzoquinone (BQ) at a level of 0.5 mM. Benzoquinone picks up electrons at a point soon after substance Q, and the vigour of the system is measured by monitoring the oxygen evolution capabilities of the thylakoids.

2.7.4.1 Types of experiment carried out to investigate copper's effects on PS II activity.

A similar progression of experiments was followed in these studies as was previously used with the thylakoids engaged in PS I transport. Thus, preincubation experiments, in which chloroplasts were kept in various copper concentrations in LSB on ice and then

sampled at intervals of time, were used and effects on both coupled and uncoupled rates assessed. Comparisons were also made of the severity of the inhibition obtained when the pretreatment period of the organelles with cupric ion was in the light and the dark. A further series of experiments, designed to investigate the relationship between the level of chlorophyll fluorescence and the inhibition caused by a particular metal dose were also performed.

2.7.5 Use of the 518 nm shift in chlorophyll absorption to investigate the possible uncoupling action of copper.

This method was employed to further investigate the possible uncoupling action of copper in isolated broken chloroplasts.

The technique is based on the principal that, during normal photosynthesis, charge seperation arises across the thylakoid membranes with a proton build-up on the inside. The existence of this charge seperation is important for phosphorylation to occur. The electrical gradient across the thylakoids has an associated electrical field and this modifies the electronic energy levels within the bulk pigment molecules (chlorophyll b, carotenoids and chlorophyll a) embedded in the membranes. This modification results in a slight shift in the absorption spectrum of the pigment molecules which then have an absorbance peak at a wavelength of 518 nm.

In practice, one illuminates the chloroplast sample with a short-duration (0.6 msec.), high-intensity light flash, sufficient to cause a "single turnover" of the photosynthetic apparatus. Charge seperation occurs and this, in turn, gives rise to the 518 nm absorption shift. The change in absorption is followed using a low intensity monitoring beam, the light passing through the sample being detected using a photo-multiplier. The rise in absorption, on illumination, is very fast (<20 nsec.), and the signal then decays away, taking from about 10 to 200 msec. This decay is connected with the movement of protons and other ions across the thylakoid membrane so as to abolish the electrical field. If the chloroplasts are uncoupled using a compound that facilitates the movement of these ions across the thylakoids, then one expects the same absorption shift, but a faster decay phase.

Because the signals from the system are rather small, and have a low signal-to-noise

ratio, a digital signal averager was employed which effectively removes the random noise component. The signal was displayed on a cathode ray oscilloscope and a permanent trace obtained using a chart recorder.

In my experiments, a chlorophyll concentration of 50 µg/ml was used, and the chloroplasts were suspended in a buffer of 50 mM tricine, made up to pH 8.3 with KOH. Absorption traces were accumulated after different times of preincubation, up to 30 minutes, in various copper treatments. Each trace obtained was the average of eight replicate flashes. A set of control traces were recorded at the outset of the experiment, and also at the end after metal treatments. This enabled a check to be made on the natural ageing of the chloroplasts. Copper treatments of 3.33, 10 and 20 ppm were employed.

Using the traces obtained, calculations were made to obtain values for the time taken for half of the absorption peak to decay away. Attempts were then made to correlate these $t_{\frac{1}{2}}$ values with the copper treatment administered.

2.7.6 Photosystem II chlorophyll fluorescence work.

In this context, fluorescence is a common phenomenon in plants whereby in vivo chlorophyll absorbs light of one wavelength and emits light of another. Isolated chloroplasts, unicellular algae etc. are suitable experimental material. One can look at the chlorophyll fluorescence yield, from a sample of defined pigment concentration, relative to a control or one can look at the kinetics of the fluorescence inhibition over time. Fluorescence can be a useful tool for looking at partial electron transfer reactions within PS II.

2.7.6.1 Methods used in assessing copper's effects on PS II fluorescence.

The apparatus employed consisted of a cuvette holder mounted within a light-tight metal box. Provision was made for bright light to be shone onto the cuvette from one side and a photomultiplier was mounted at right angles to the lamp to collect light emitted from the sample. This incident light passed through a blue filter combination (Schott BG 38 and BG 18) and the emitted light through two red filters (RG 665 cut off filter and Balzer 687 interference filter). The current from the photomultiplier, due to

the fluorescence emission, was passed through a 10 KΩ load resistor and the resulting voltage displayed as a trace on a Rikadenki variable-speed chart recorder. The basic medium used was again LSB and the standard chlorophyll concentration used, 7.6 µg per ml, was purposely kept low so as to ensure light saturation. Opening of a shutter caused instantaneous illumination of the sample and a trace was produced showing fluorescence yield.

In most experiments, a chloroplasts suspension was placed in a cuvette in the apparatus and then left for one minute in the dark to achieve temperature equilibration. In some experiments, other additions were also present at this point. A small rubber diaphragm in the top of the apparatus enabled one to inject small volumes of reactants into the cuvette without allowing light to get in. A 100 µl syringe was always employed for this purpose as it could be filled with solution from the cuvette and the whole volume could then be expelled suddenly to help to ensure rapid and efficient mixing of the reactants with the chloroplasts.

In most experiments, either (i) copper was present with the chloroplasts from the outset and the light was subsequently turned on. Here, the initial level of fluorescence yield after a treatment could be obtained, essentially at time zero, and this was then compared to a separate control, or (ii) a reactant mixture with chloroplasts was allowed to reach a steady-state fluorescence level, and then the copper was added. This method gave a true "internal" control for every sample.

In several experiments, once an effect of copper at a particular level had been demonstrated, various artificial electron donors were then added in an effort to overcome the fluorescence inhibition. The various artificial electron donation systems employed were as follows :

(i) p-phenylenediamine ($C_6H_4(NH_2)_2$) at 33 µm with sodium ascorbate at 330 µM

(ii) 1,5-diphenylcarbazide ($C_6H_5.NH.NH)_2CO$ at 5 mM (in acetone)

(iii) Hydroxylamine (monohydrochloride) (NH_2OH) at 5 mM

(iv) Manganese chloride, ($MnCl_2$), at 1 mM

In many samples, magnesium chloride was present at 5 mM. Magnesium, a divalent cation, boosts the fluorescence yield to a higher level. DCMU, again at 5 μM, was also often present in the reaction medium. Ascorbate, at 330 μM, was tried as an electron donor on its own, and controls were run with distilled water and acetone as these were used as solvents for the other additions.

Several experiments were run using chloroplasts modified by a "tris buffer wash". These were prepared by resuspending the pellets, obtained after the centrifugation step of the extraction proceedure, in a few mls of 0.8 M tris at pH 8 for 15 minutes. They were then reseparated by centrifugation, suspended in single-strength LSB and used as normal. It was usually necessary to pass tris-washed chloroplast material once or twice through a ground-glass homogeniser in order to obtain a usable uniform suspension.

Comparisons were made of the degree of fluorescence boosting that could be gained with normal and tris-treated chloroplast material.

2.7.6.2 Experiments conducted to investigate the metal inhibition of PS II fluorescence.

The experiments carried-out can be summarised under the following groupings :

(i) Sensitivity of fluarescence to inhibition by various levels of copper and cadmium.

Measurements were made of final steady-state fluorescence yields after copper and cadmium treatments. Fluorescence inhibition in normal versus tris-treated broken chloroplasts after two minutes exposure to copper was investigated. The effects of up to 1.1 mM copper on fluorescence after two minutes preincubation, and the effect of then adding DCMU, was also studied.

(ii) Relevance of cation (Mg^{2+}) presence in chloroplast susceptibility to fluorescence inhibition by copper.

Measurements of initial fluorescence yields after various metal treatments were made with and without $MgCl_2$ present (at 5 mM) and the results expressed as a percentage of controls.

(iii) Relevance of DCMU presence in chloroplast susceptibility to fluorescence inhibition by copper.

As in (ii) initial chloroplast fluorescence yields were measured in various metal treatments both with and without DCMU (at 5μM) being present.

(iv) The effectivity of various PS II artificial electron donors as fluorescence-stimulating agents in normal and tris-treated chloroplasts.

Final fluorescence yields after the addition of hydroxylamine, manganese chloride, diphenyl-carbazide and phenylenediamine plus ascorbate (all with DCMU subsequently added) were found for normal and tris-washed chloroplasts.

(v) The use of various electron donors in attempts to restore fluorescence levels after copper inhibition.

Attempts were made to restore fluorescence in tris-washed chloroplasts using PD and Asc. after treatment with up to 1.058 mM copper. Similar experiments with hydroxylamine used normal and tris-washed material and the same proceedure was followed with diphenylcarbazide, but then cadmium was also used. Manganese chloride was used with normal chloroplasts pretreated with both metals.

(vi) Fluorescence induction.

Fluorescence induction curves, showing the first few seconds of response after illumination of the chloroplasts, were accumulated at intervals over a twelve minute preincubation period in various levels of copper treatment up to 5 mM.

2.8 Chlorella fluorescence work.

2.8.1 Methods employed in Chlorella fluorescence work.

The fluorescence apparatus employed was exactly as used for the previous chloroplast work. The basic reaction solution used to suspend the cells was full Chlorella medium, as it was desirable to keep the cells as near normal physiologically, as possible. Cells were used at a standard pcv of 0.15%.

The types of fluorescence experiment conducted fell into two categories :

(i) Fluorescence induction studies.

Chlorella cells were dark preincubated for various times in copper levels of up to 30 mM and were then subjected to instantaneous illumination. The presence of DCMU was investigated as a separate factor in some cases.

(ii) Use of artificial electron donors.

Hydroxylamine and phenylenediamine plus ascorbate were investigated as possible fluorescence restoratives after a copper treatment.

CHAPTER III

RESULTS

3. RESULTS

3.1 Work with Chlorella pyrenoidosa.

3.1a Growth experiments using copper sulphate.

(i) Copper toxicity in normal full medium.

In order to gain an idea of the relevant scale of necessary copper additions to affect Chlorella growth, a preliminary run of this series of ecperiments was conducted in aerated dreschel bottles. The growth traces obtained over 11 days are depicted in Figure 1. The 1 ppm (16 μM) Cu treatment had no appreciable effect on culture development, while 4 ppm depressed it significantly. The cells in 30 ppm Cu (476 μM) did not grow at all.

In a second run of the same type of experiment, conducted in the incubator, the growth traces produced were as shown in Figure 2. Figure 3 replots the data as a % of the control values. The figures taken together indicate that there is a graded response of Chlorella cultures to increasing levels of copper addition. Copper levels of 0.5, 1.0 and 2.0 ppm had no significant effect on culture development over the experiment period (210 hours). Levels of 5.0, 10.0, 20.0 and 50.0 ppm had an increasingly serious effect with the cells in the highest metal treatment hardly growing at all. There was, however, a very slight rise in culture density in even the 50 ppm treatment over time. The 20 and 50 ppm treated cultures were significantly different from controls from the second sampling time onwards. It is likely that statistical significance is largely lacking for the other treatments because, as time progresses and the poisoning begins to take effect, the biological variability of the cultures also increased, giving rise to larger and larger 95% confidence intervals. Table I summarises the results of the monitoring of the copper levels in solution during the experimental period.

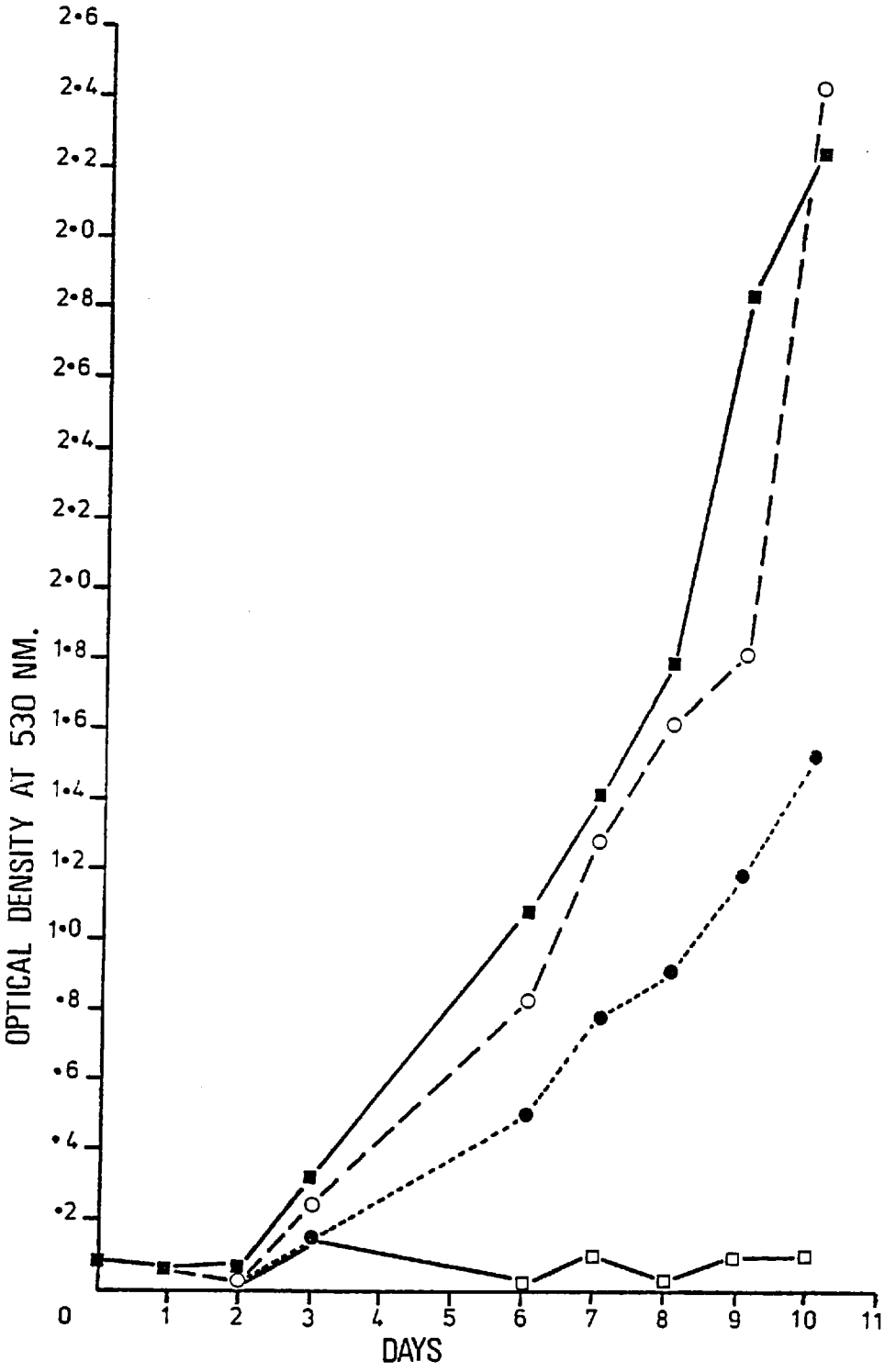


Figure 1. Growth of Chlorella cultures in aerated dreschel bottles containing normal medium and copper additions of 0 (■), 1 (○), 4 (●) and 30 ppm copper (□).

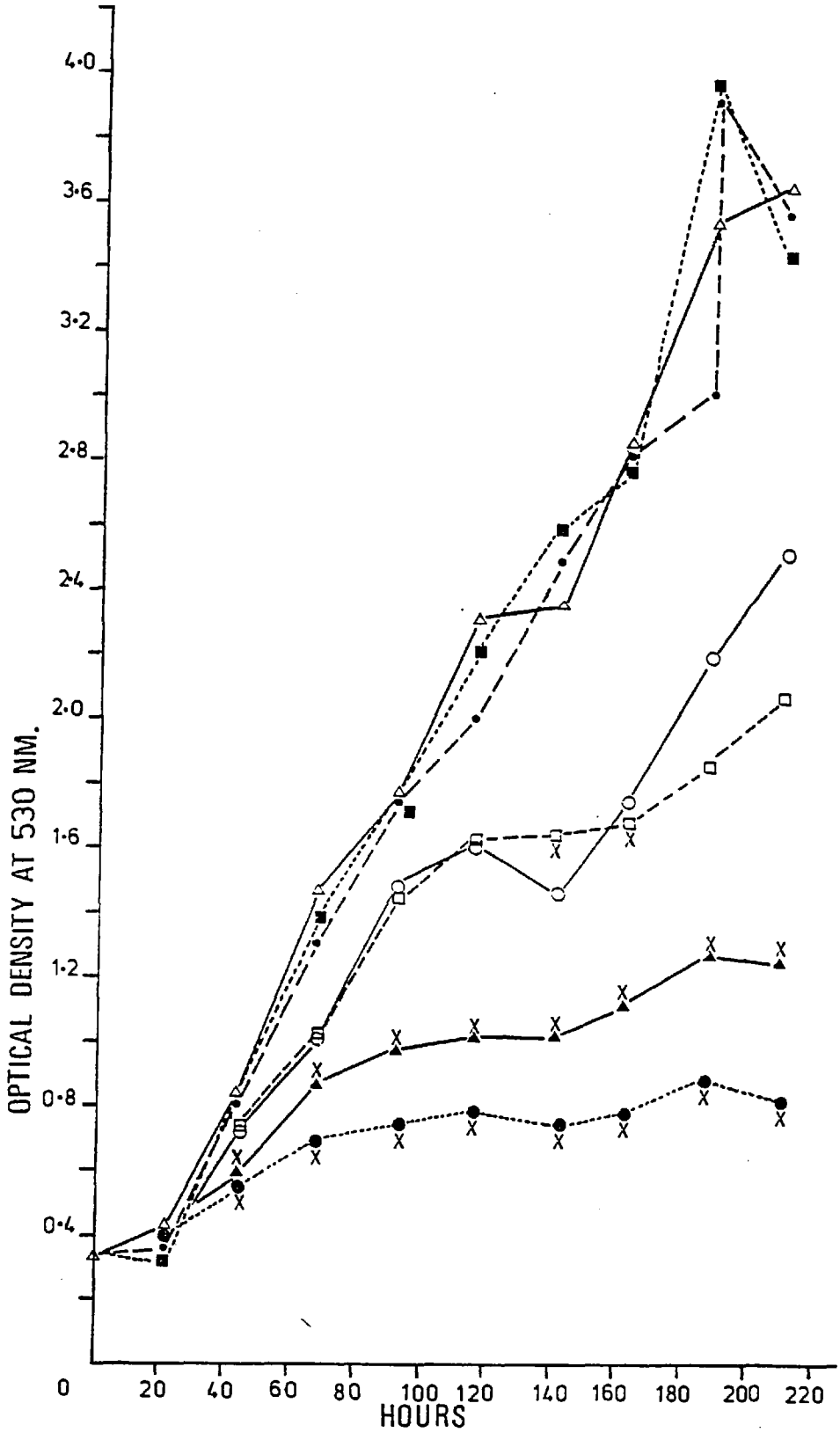


Figure 2. Growth of Chlorella cultures in media containing 0 (△), 0.5 (■), 2 (•), 5 (○) 10 (□), 20 (▲) and 50 ppm copper (●). X denotes a significant difference from controls (no overlap of 95% confidence limits).

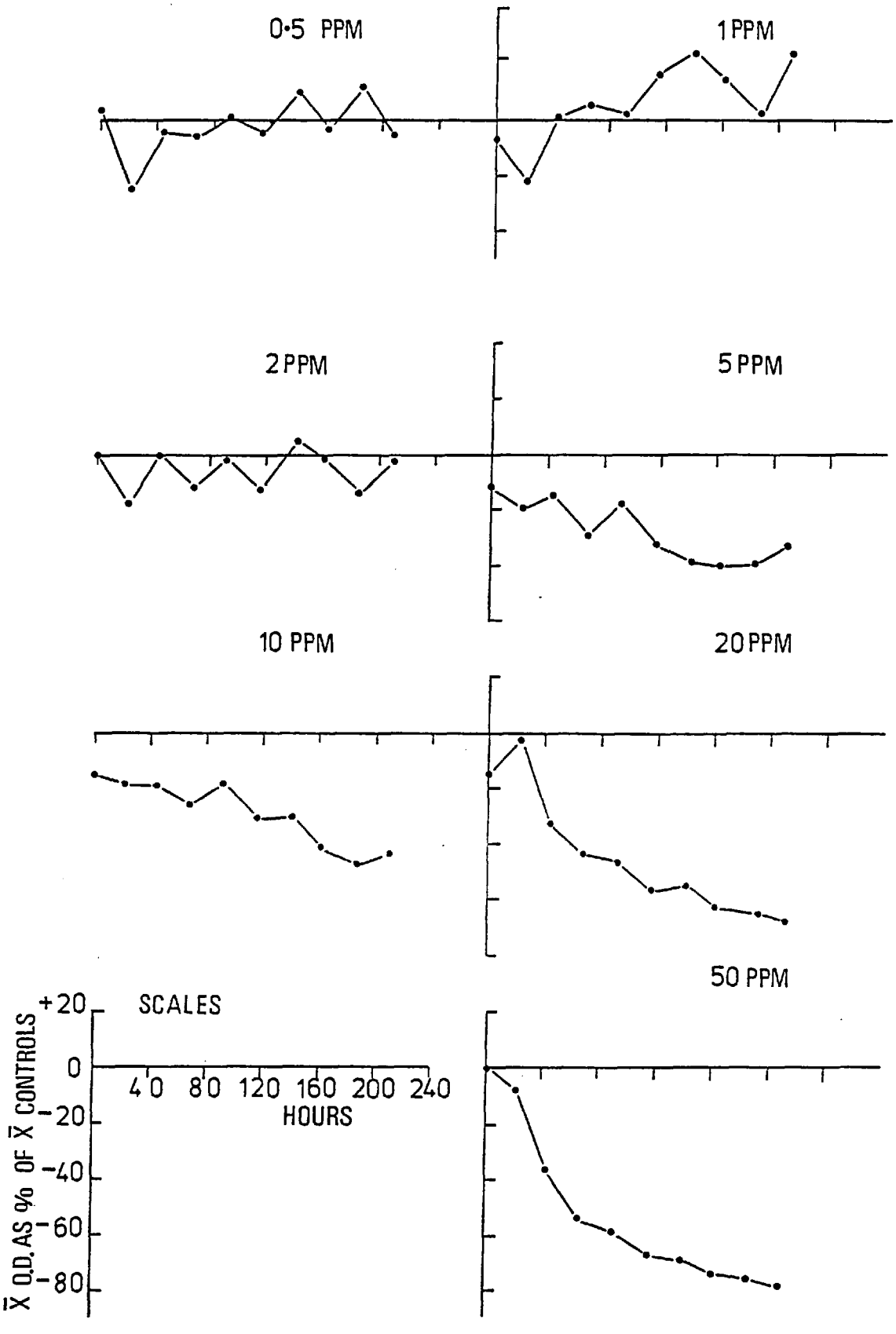


Figure 3. Growth of Chlorella cultures subjected to various copper regimes. Results are plotted as a % of mean control values.

TABLE I Levels of copper in the medium of various metal treatments over the experimental period. Except for the initial values, which were single samples, the results are the mean of 4 replicates ± the 95% confidence intervals.
* signifies a significant difference from the control value.

		ppm copper in media samples					
Sampling times (hrs)→ Treatment ↓	initial pre-inoculation	0	22	68	116	162	210
Control	$\bar{x}$	0.90	1.31	0.68	0.86	0.51	0.31	0.34
	CI		0.15	0.51	0.04	0.14	0.08	0.08
0.5 ppm	$\bar{x}$	1.55	1.33	0.81	0.80	0.63	0.54*	0.40
	CI		0.33	0.23	0.11	0.10	0.08	0.11
1.0	$\bar{x}$	1.50	1.08	1.09	1.08	0.94*	0.78*	0.58*
	CI		0.40	0.12	0.45	0.25	0.08	0.10
2.0	$\bar{x}$	2.80	1.75	1.58*	1.35	1.29*	0.98*	0.65*
	CI		0.38	0.19	0.64	0.23	0.15	0.20
5.0	$\bar{x}$	5.04	4.92*	4.61*	5.13*	4.58*	2.43*	3.45*
	CI		0.34	0.51	0.17	0.35	1.24	0.91
10.0	$\bar{x}$	8.50	8.63*	9.38*	8.50*	8.63*	7.63*	5.88*
	CI		2.28	1.19	1.45	1.36	0.40	1.36
20.0	$\bar{x}$	15.00	11.75*	12.88*	11.88*	12.00*	11.13*	11.25*
	CI		4.23	1.00	0.76	2.05	1.36	1.52
50.0	$\bar{x}$	30.50	24.88*	21.13*	10.75*	9.38*	9.38*	9.75*
	CI		2.78	2.28	0.80	0.76	0.76	0.46

It can be seen that, in most cases, the levels of copper in the media were lower than predic ted from the additions of standard used. This is because the data refers to copper in solution in the medium only. It is very likely that, apart from the copper uptake by the algae themselves, a certain proportion of it is also lost from solution as insoluble precipitates, perhaps as the carbonate, which are not included in the analysed samples as they are removed by the centrifugation step. The main point is therefore perhaps that the cells are, in fact, more sensitive to copper poisoning than might have been assumed from the original additions. This point is perhaps particularly valid at the higher metal concentrations where less of it is retained in solution.

A graph, Figure 4, was prepared, showing the mean deviation over the whole experimental period of Cu-treated culture O.D. values from mean controls against overall average copper levels in solution. A fairly linear relationship (negative slope) was obtained; the greater was the average medium copper level over the 210 hour period, the greater was the growth inhibition.

(ii) Copper toxicity in relation to level of chelator presence.

This work aimed to compare copper's toxicology under various levels of EDTA presence because chelators are present in many nutrient media and their inclusion has been reported to have a large effect on the effectivity of the metal as a poison.

Table II gives the results of an experiment in which the effects of copper treatments on Chlorella cultures, growing with and without EDTA present, were assessed. The normal level of micronutrients was present in the EDTA-free cultures; the Hutner's solution employed for these replicates was as normal except that it contained no chelator.

TABLE II Chlorella cutlure growth in various copper treatments, with and without EDTA being present.

TREATMENT ($\bar{x}$ measured Cu level over the whole experimental period.)	Optical densities (at 530 nm) at various sampling times (hrs.)							
	0	22.5	73.5	94.5	118.0	142.5	166.5	189.5
.47 ppm with EDTA	.20	.26	.40	.50	.62	.78	.99	1.23
.00 ppm, no EDTA	.21	.28	.42	.48	.70	.89	1.10	1.44
3.31 ppm with EDTA	.18	.25	.43	.46	.72	.91	1.10	1.40
.22 ppm, no EDTA	.21	.30	.35	.43	.60	.80	.65	.73
3.71 ppm with EDTA	.18	.24	.48	.53	.77	1.01	1.12	1.72
.67 ppm, no EDTA	.20	.26	.31	.37	.44	.42	.44	.45

(Treatments bracketed together had the same copper additions, but less was retained in solution with no EDTA present. Results are the mean of four replicates.)

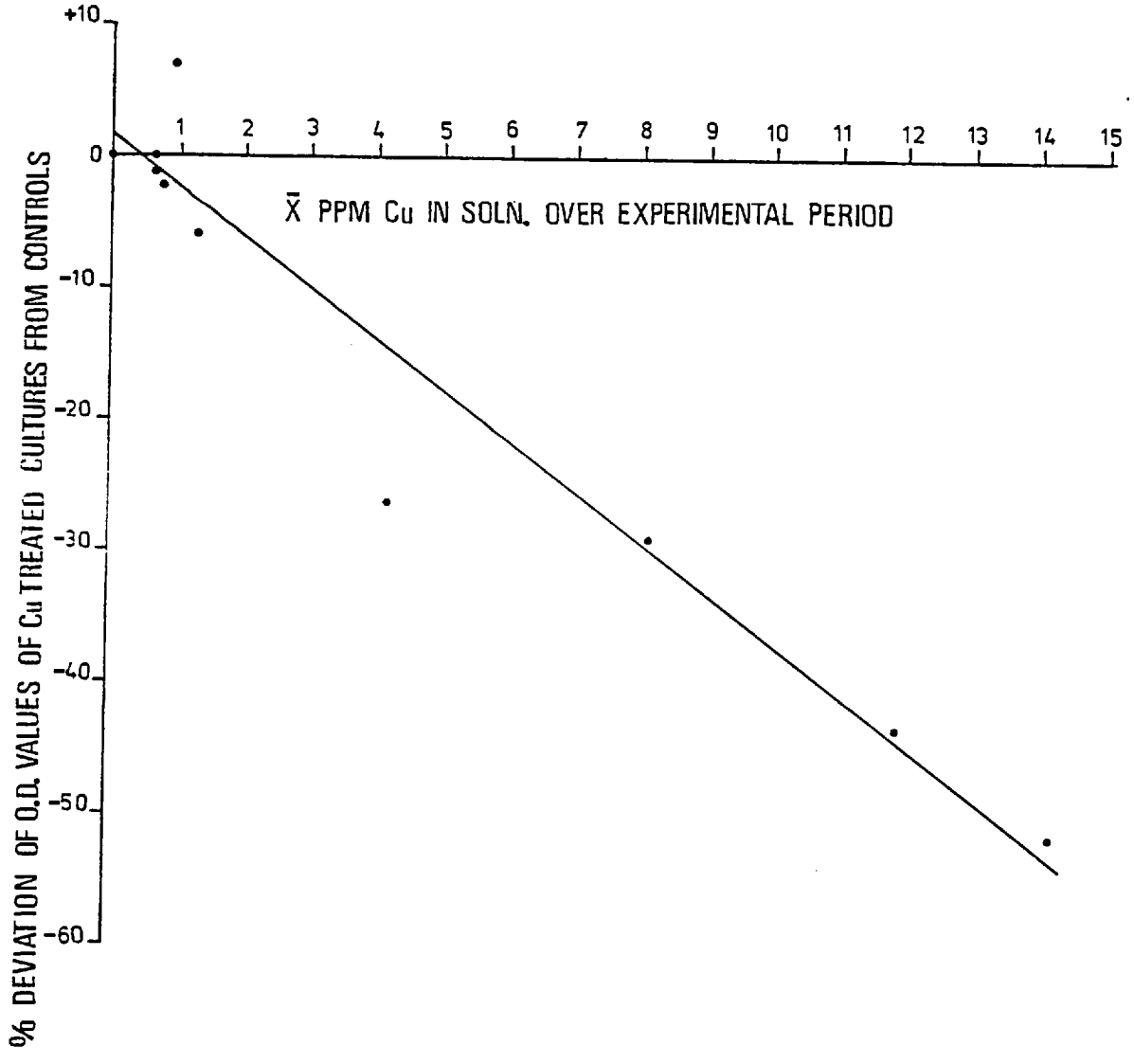


Figure 4. Mean % deviation from control values, over a 210 hour experimental period, of the O.D. values for Cu-treated Chlorella cultures, plotted against the mean Cu Concentration in solution over that time.

The results therefore demonstrate that the lack of EDTA had no adverse effects on the growth of control cultures, but that its presence in the other two treatments led to a definite reduction in copper toxicology; this was the more striking because it was in the cultures with chelator present that the metal levels were highest. With no EDTA in the medium, very low levels of copper could be seen to have a severely inhibitory effect on culture growth.

Table III summarises some further results of an experiment in which 15 ppm copper was added to media containing a range of chelator levels. Less copper was retained in solution as lower levels of chelator were employed. Growth of the Cu-treated and control cultures was monitored over 195 hours.

TABLE III Chlorella culture growth in media containing various levels of chelator addition. 15 ppm copper was used as the metal treatment in all cases.

EDTA level (mM)	Treatment ($\bar{x}$ initial Cu ppm)	$\bar{x}$ optical densities (at 530 nm) at following sampling times (hours)															
		0	6	23	28	30	47	52	54	71	76	95	102	122	146	167	195
.27	0.92 control	.24	.22	.36	.35	.37	.44	.47	.50	.63	.64	.74	.80	.83	1.06	1.15	1.25
.27	16.00	.24	.24	.30	.25	.26	.27	.27	.30	.34	.29	.32*	.33*	.33*	.43*	.44*	.56*
.10	0.39 control	.24	.22	.34	.33	.34	.38	.43	.43	.57	.50	.62	.68	.81	.88	.98	1.14
.10	11.35	.23	.23	.28	.23	.25	.25	.26	.26	.31	.26*	.29*	.29*	.31*	.37*	.36*	.39*
.01	0.20 control	.26	.23	.36	.34	.34	.42	.45	.45	.58	.57	.70	.74	.85	.94	1.02	1.13
.01	6.10	.23	.22	.27	.22	.23	.23	.23	.23*	.28*	.24*	.26*	.25*	.27*	.31*	.30*	.34*
.001	0.08 control	.23	.22	.35	.33	.35	.38	.38	.42	.53	.49	.64	.68	.83	.92	1.00	1.12
.001	4.25	.25	.23	.26	.23	.23	.23	.23	.25	.30	.24*	.26*	.25*	.27*	.32*	.30*	.34*
$\bar{x}$ of all 4 sets of controls		.24	.22	.35	.34	.35	.41	.43	.45	.58	.55	.67	.72	.83	.95	1.04	1.16

(Because of the number treatments, there were only two replicates for each sample in this experiment. x ≡ point at which results are less than $\frac{1}{2}$ $\bar{x}$ controls).

Again, the various control values indicated that the progressive EDTA deficiency itself had not had a very serious effect on growth. A copper treatment of 4.25 ppm (.001 mM EDTA) had a greater inhibitory action on growth than did 16.00 ppm with EDTA present at 0.27 mM.

Figure 5 depicts the growth traces for an experiment, similar to the above except in that the range of Cu concentrations was lower (additions of 10 ppm instead

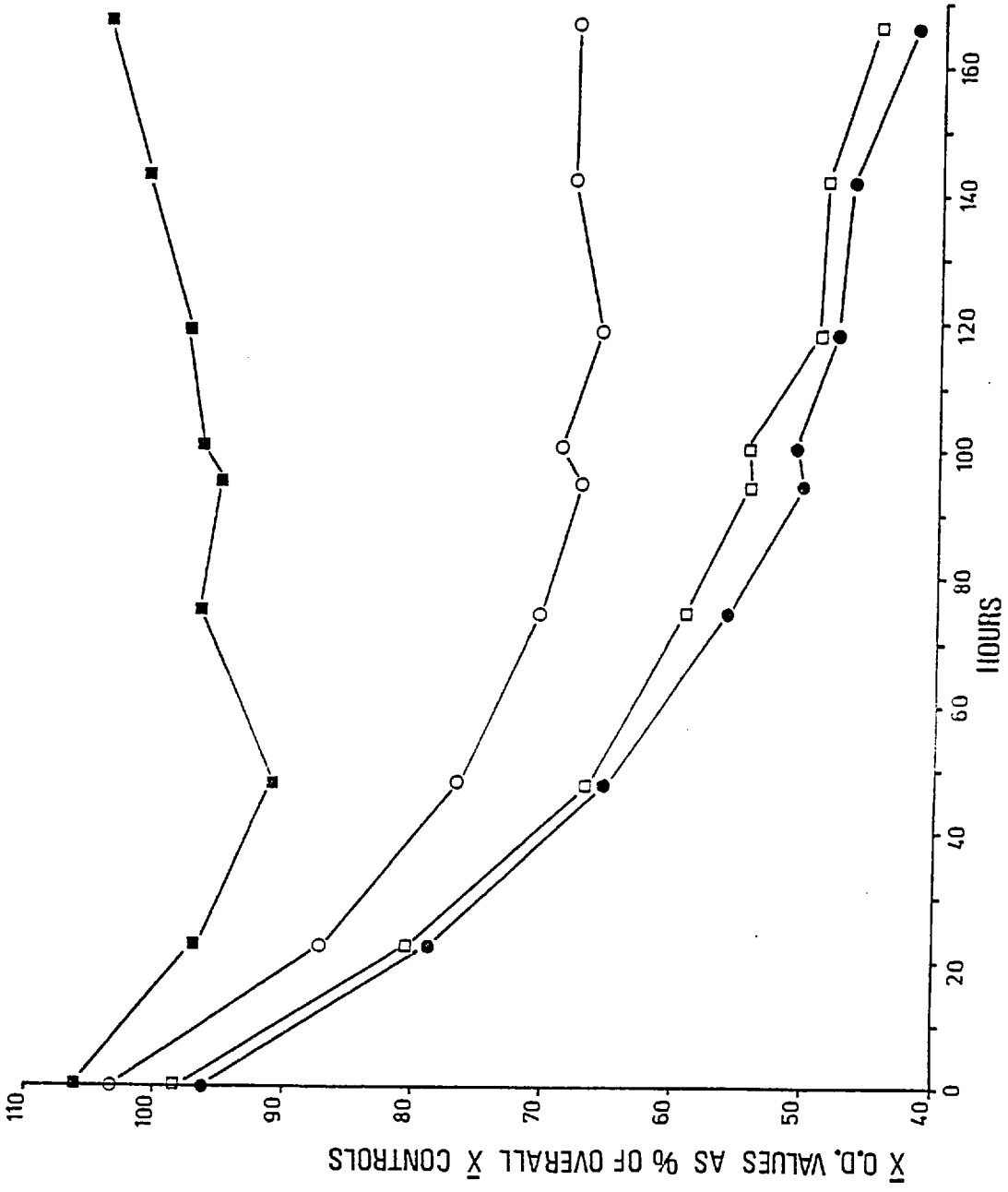


Figure 5. Growth of Chlorella cultures in media containing various levels of EDTA. 10 ppm copper was added in each case and results are expressed as a % of mean controls. 0.27 mM EDTA, 6.4 ppm Cu retained in solution (■), 0,10 mM EDTA, 5.0 ppm Cu (o), 0.01 mM EDTA, 3.2 ppm Cu (□), 0.001 mM EDTA, 2.0 ppm Cu (•).

of 15 ppm as above). This aimed to demonstrate a more graded response to decreasing chelator levels. None of the various control results lagged behind true controls (0.27 mM EDTA) by more than 6%. The traces are therefore expressed as a % of overall average controls.

The most interesting point to be gleaned from these experiments is that, although the lowering of EDTA levels results in less of the applied copper being retained in solution, the toxicity of that reduced fraction that does remain biologically available is similarly much increased. Thus, one can obtain a succession of results whereby growth suppression is increasingly severe with decreasing chelator levels while, at the same time, copper levels in solution are also falling. For example, Table II showed that cells could grow quite successfully with 3.71 ppm copper when EDTA was present, but that, with no chelator, growth was severely suppressed, although the copper levels in solution had then fallen to only 0.67 ppm.

Table III and figure 5 demonstrate the same idea, but emphasise that there is a graded response, whereby the lower the chelator level, the greater is the growth suppression, again despite attendant reductions in dissolved metal. The grading of the response is probably more marked in figure 5 than in Table III because the prevaling copper levels were lower.

Table IV gives growth data for a set of four cultures grown with either control or $1/10^{th}$ normal levels of micronutrient (and therefore EDTA) additions.

TABLE IV Chlorella culture growth in media with either normal or $1/10^{th}$ normal micronutrient (and therefore EDTA) additions. (*signifies a statistical difference from the relevant set of controls.)

$\bar{x}$ Cu level present over the experimental period (ppm)	micronutrient presence	Optical densities (at 530 nm) at following sampling times (hrs.) 0	25	72	99	123	144	171	193
0.75	normal $\bar{x}$	.23	.32	.69	.88	1.02	1.07	1.40	1.59
	CL	.01	.05	.27	.30	.34	.27	.44	.50
4.94	normal $\bar{x}$	.22	.31	.87	1.20	1.27	1.34	1.44	1.47
	CL	.02	.07	.41	.63	.66	.69	.78	.62
0.31	$1/10$ normal $\bar{x}$	.20	.27	.76	.86	.90	.98	1.13	1.10
	CL	.02	.15	.28	.31	.29	.20	.24	.20
1.49	$1/10$ normal $\bar{x}$	.20	.26	.32*	.32*	.38*	.46*	.49*	.59*
	CL	.03	.06	.13	.15	.13	.19	.20	.20

These results indicate that, with a reduced micronutrient (i.e. EDTA) presence, a $\bar{x}$ level of only 1.49 ppm copper can severely (and significantly) inhibit Chlorella growth, while, with the normal level of micronutrients, even 4.94 ppm did not seem to supress culture development at all.

(iii) Effects of time of exposure to copper on Chlorella growth.

Figure 6 compares Chlorella culture growth after various times of exposure to either 0, 5 or 40 ppm copper. No real difference can be seen between the traces after 10, 30 or 120 minutes exposure. After 240 minutes, however, there seemed to be a trend for the Cu-treated cells to be growing slightly slower than controls. It is likely that the washing of the cells in distilled water and then 1 mM EDTA, between exposure to the metal and subsequent growth monitoring, served to remove much of the adsorbed and/or absorbed copper, apparently allowing cell recovery.

Table V gives the results for a similar experiment, except that, in this case, the EDTA wash was omitted. The initial copper levels in the medium were, as analysed, 0.92 ppm (controls) and 24.80 ppm.

TABLE V The effects of the time of exposure to a copper treatment of 24.80 ppm on Chlorella culture growth.

Treatment	Optical densities (at 530 nm) at following sampling times (hours)										
	0	3.7	20.9	26.2	45.2	52.1	72.1	95.6	117.1	145.1	190.1
$\bar{x}$ of all controls	.07	.03	.10	.05	.09	.12	.26	.36	.45	.72	.92
60 mins. exposure to 24.80 ppm	.05	.03	.10	.05	.07	.11	.17	.35	.46	.67	.88
150 mins. exposure to 24.80 ppm	.09	.03	.10	.04	.08	.10	.14	.26	.40	.64	.82

Again, the effects on growth were anything but dramatic, although there was a definite tendency for the Cu-treatments to have an inhibitory effect on growth. The 150 minutes exposure to copper seemed to cause greater suppression than did 60 minutes. It seems likely that more clearly-defined growth inhibitions were not obtained because, despite the omitting of the EDTA wash, chelator was still present in the growth medium used.

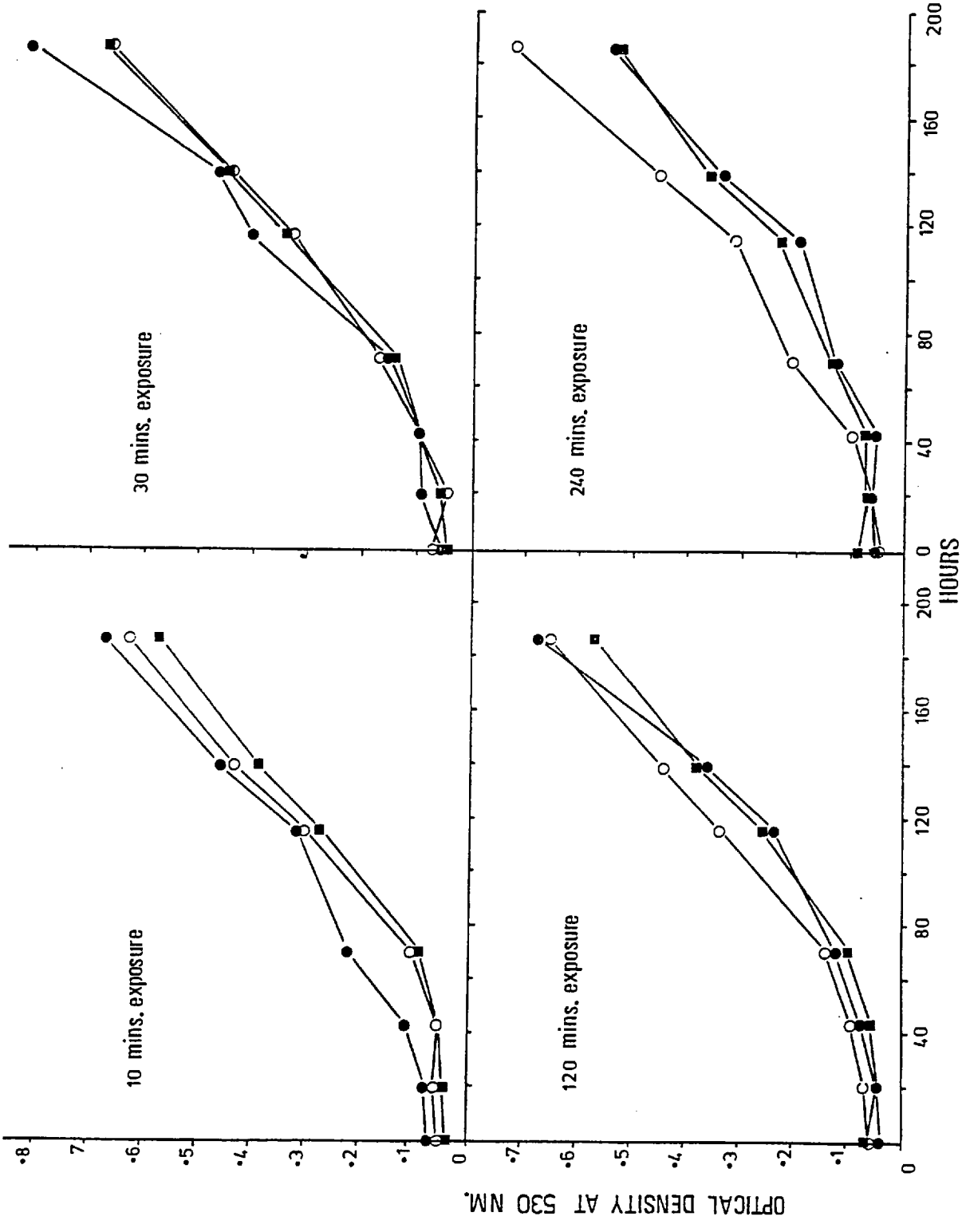


Figure 6. Growth of Chlorella cultures after various times of exposure to either 0 ppm (○), 5 ppm (■) or 40 ppm Cu (●).

3.1b Work with release glass B240177.

In order to investigate the capability of copper to act as a growth inhibitor when supplied in a form other than as the sulphate, experiments were conducted using a RG. This method of application should result in a metal dose that remained at a fairly constant level over extended periods, and it should contrast with more conventional additions where an initial dose level, perhaps high, thereafter declined in concentration.

(i) Release of copper into various media.

Figure 7 demonstrates Cu-release from B240177 into Chlorella medium and distilled water. The background copper, always present in the growth medium, has been subtracted. Allowing for the few odd results that help to distort the overall picture, no real difference can be seen between the release into the two samples. There seems to have been a relatively high initial rate of metal release over the first 3 to 4 hours, but this thereafter declined with time.

Figure 8 depicts a similar set of results, this time to follow metal release into distilled and sea water. The main point is that the release into the two was very similar up to the six or seven hour point, but thereafter there was an increasing divergence with the levels in distilled water soon far outstripping those in the saline medium. The levels in sea water seemed to becoming steady at around 0.6 ppm Cu, whereas the concentration in distilled water, at the end of the experiment, was 2.57 ppm and apparently still rising. The explanation for this difference could either be that the rate of release of metal is greater in distilled water than in sea water or it may be that, in the latter, a largish portion of the metal may be lost from solution as insoluble forms. The distilled water was included only as a blank, against which release into the other media could be compared. The pH of the distilled water was invariably lower than in either Chlorella medium or the sea water, and this undoubtedly had a bearing; in this experiment, the mean pH of this distilled water over the experimental period was 5.04, whereas the value for sea water was 7.67.

In further work, the relevance of chelator presence on copper release into, and retention in, solution was investigated. Figure 9 shows copper levels in full Chlorella medium with and without EDTA present. There was a fairly consistent trend for the medium minus chelator to have the lower metal concentrations; it was difficult to

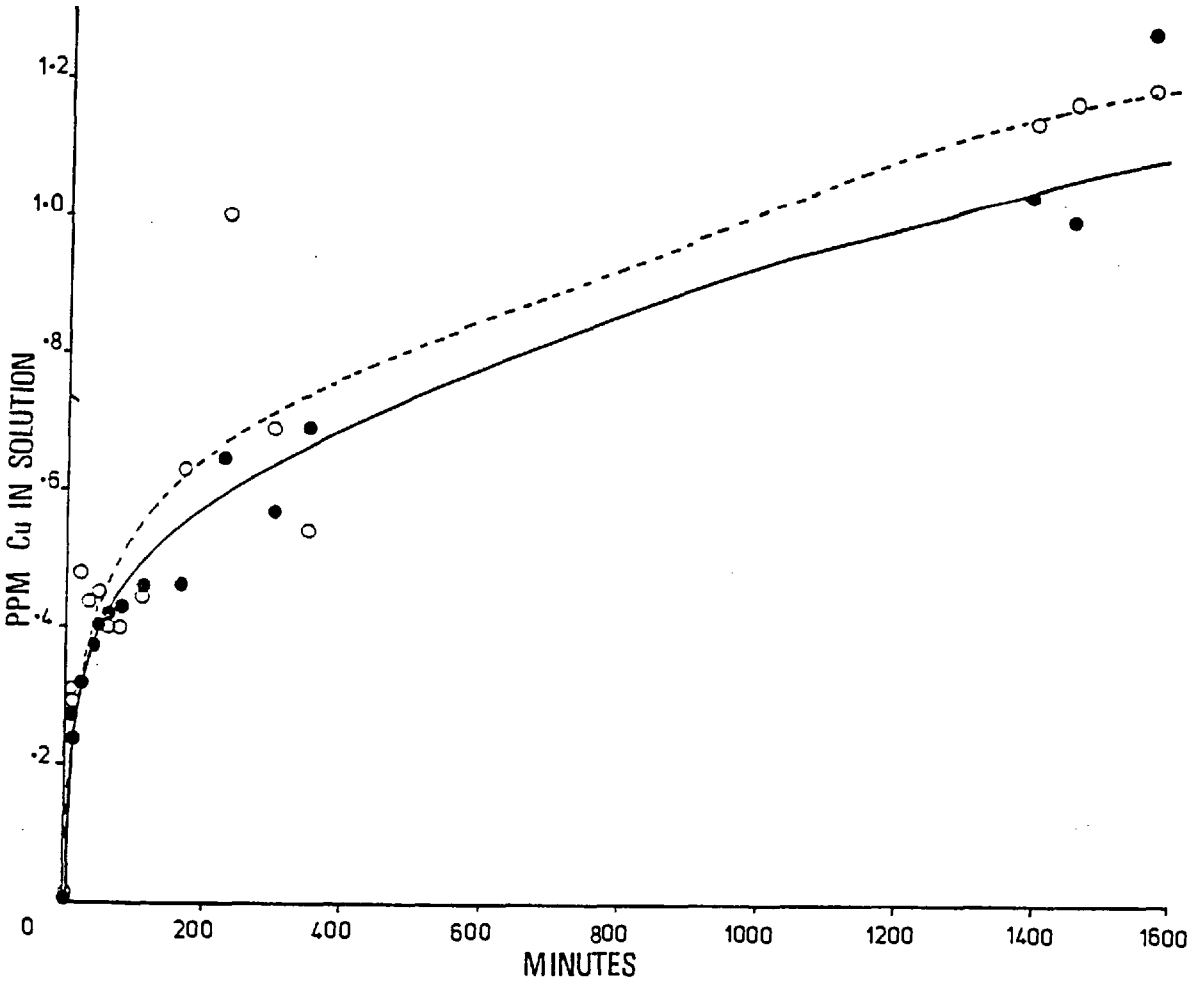


Figure 7. Copper release from Glass B240177 (present at 2g/L) into Chlorella medium (○) and distilled water (●) over time.

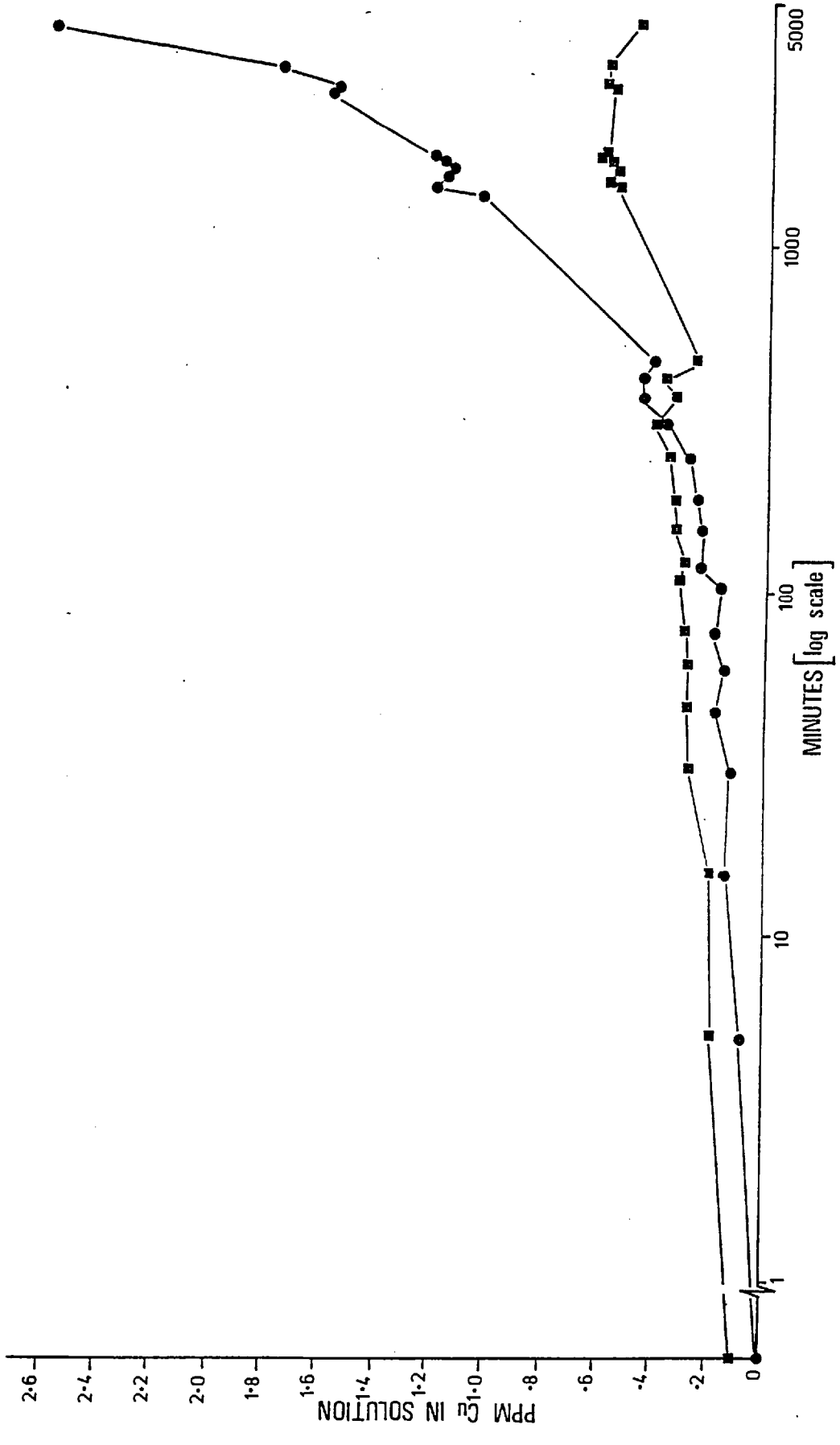


Figure 8. Copper release from Glass B240177 (present at 2g/l) into distilled (●) and sea water (■) over time.

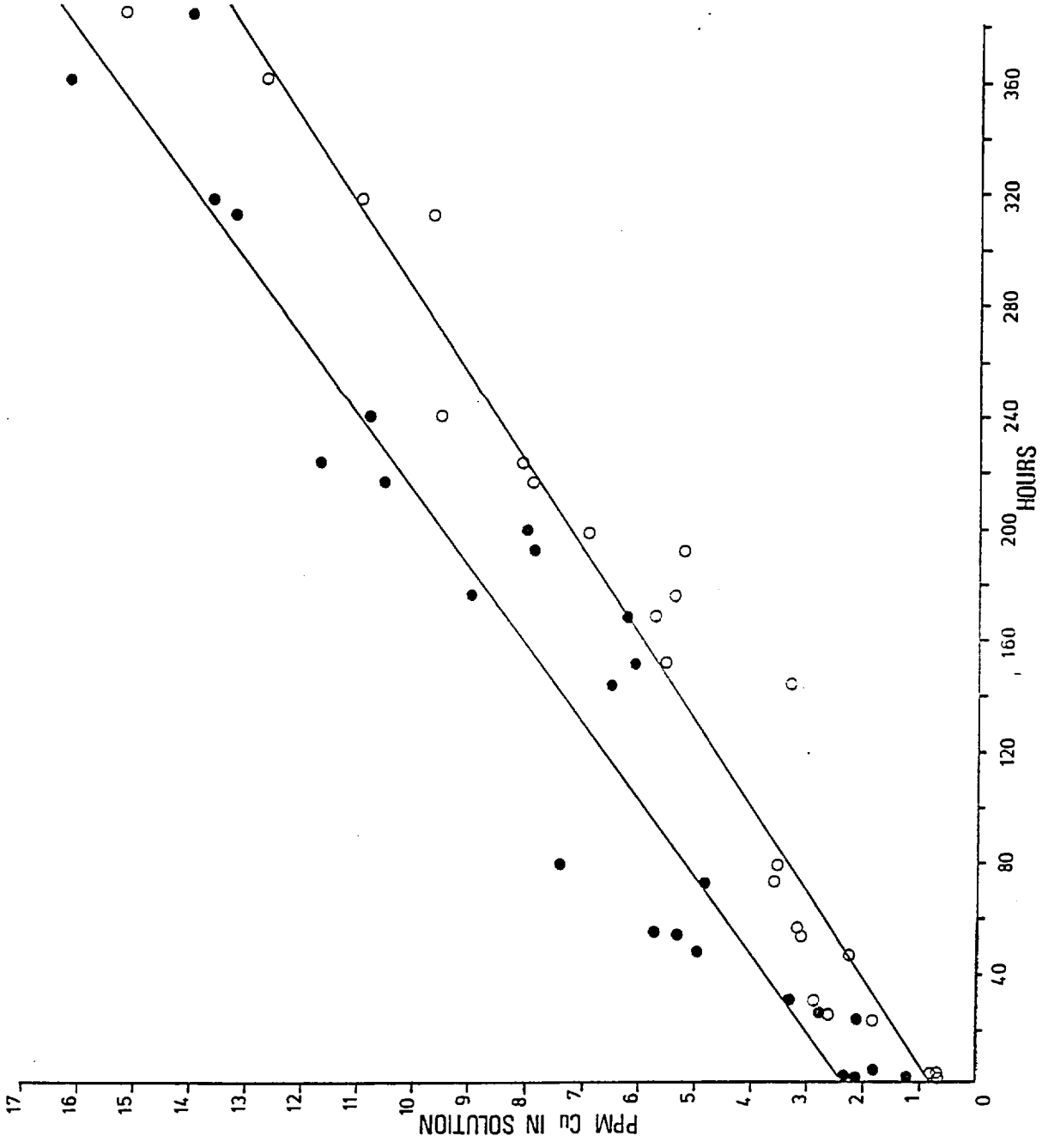


Figure 9. Copper release from Glass B240177 (present at 2g/l) into full medium with (•) and without (○) chelator present. The mean pH of the minus EDTA treatment was 5.423 (± 0.265, standard deviation) over the whole experimental period, while the corresponding value with EDTA was 5.968 (± .194).

decide if this was because of increased precipitation or a different release rate, as the medium minus EDTA had a pH consistently $\frac{1}{4}$ to $\frac{1}{2}$ pH unit lower than that of the medium with chelator.

The above trend was demonstrated even more clearly when release into a medium lacking both EDTA and micronutrients (only chelator was lacking in the above example) was compared to that into full medium (figure 10). Here, there was an even greater divergence of the graph traces with the line for the full medium finally indicating a copper level more than twice that for the micronutrient-free sample.

It is likely that the rate of release of metal into the two media was probably approximately the same, both in the case of with and without Hutners and with and without EDTA alone. The data probably therefore confirm the opinion that less copper can be held in solution when no chelator is present. The lack of micronutrients, as well as of chelator, exaggerated this result.

The above work, which had given an idea of the sort of levels of release glass application that were relevant to growth and uptake studies, was followed by similar work involving Chlorella cells. To investigate whether their presence might affect the rate of release, a comparison was made of the build-up of copper in solution in media with and without cells present. Figure 11 demonstrates the copper levels obtained and plots them alongside the growth of the cells, as monitored over the experimental period. The traces show that the culture did not grow significantly, and also that there was a trend for the medium with cells present to have higher copper levels in solution. This trend was fairly consistent throughout, but gradually become more pronounced with time. This result was confirmed by an experiment in which release into media containing different initial packed cell volumes was investigated. Figure 12 illustrates that higher metal levels were always associated with the higher cell inoculum.

(ii) Growth experiments.

The first full Chlorella growth experiment using a release glass was conducted with levels of 0.4, 0.8 and 2.4 g per 200 mls culture medium. A treatment of 5 ppm, supplied as $CuSO_4 . 5H_2O$, was also included for comparison. Figures 13 and 14 summarise both the growth data for the cultures and the copper levels in the media over time. The growth traces show that there were no observable differences at all

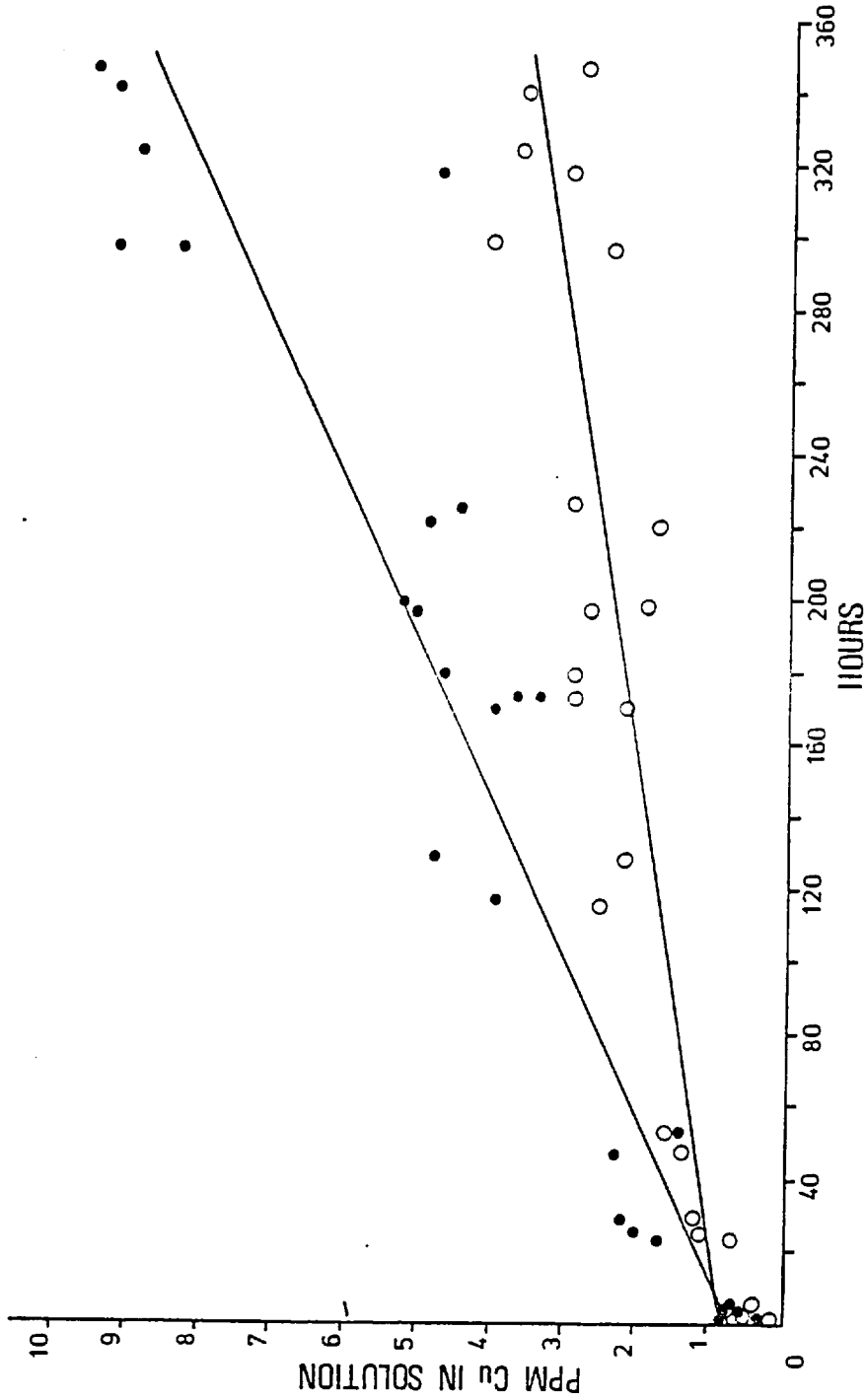


Figure 10. Copper release from Glass B240177 (present at 4g/l) into media with (•) and without (o) Hutner's micronutrients present.

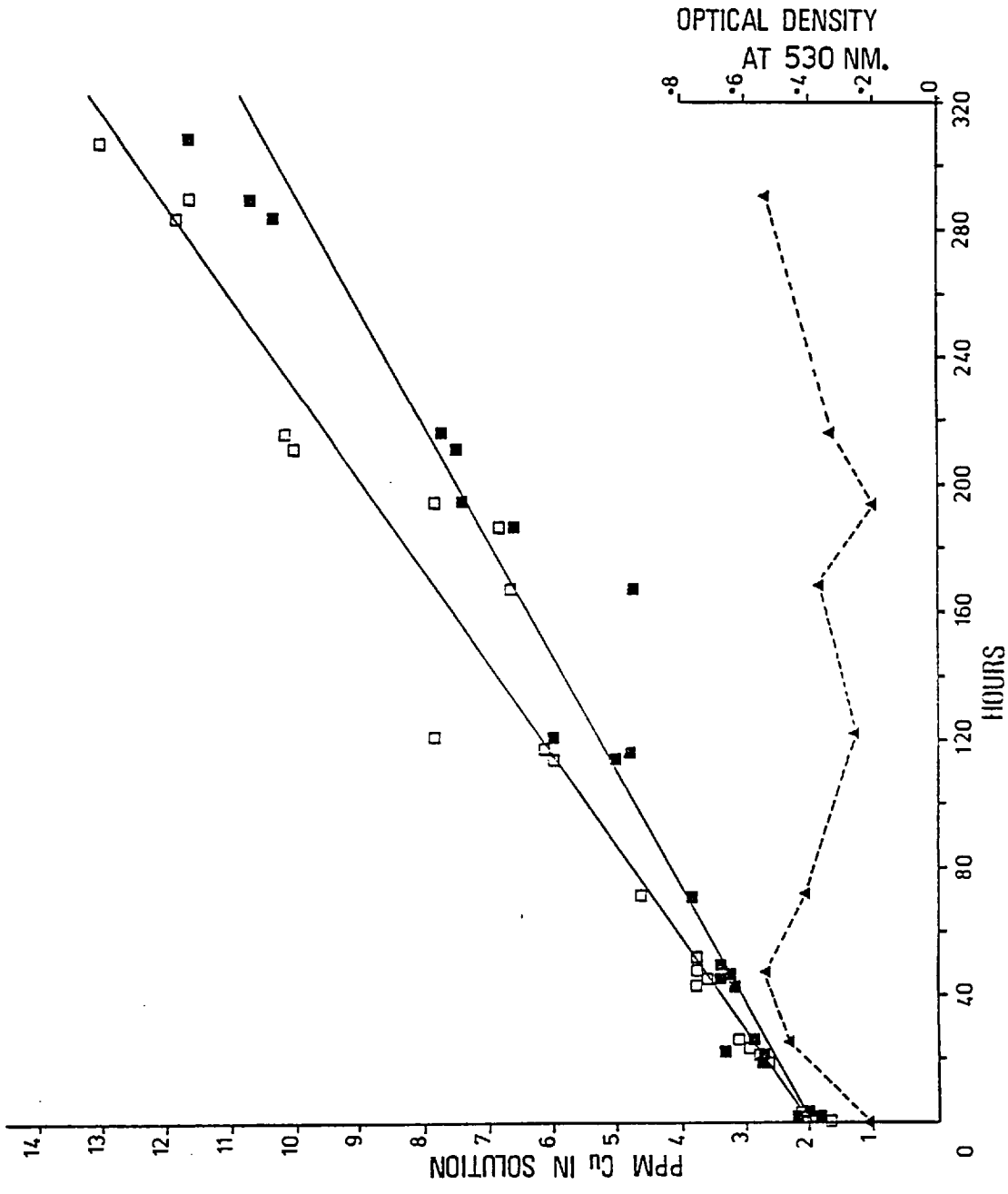


Figure 11. Copper release from Glass B240177 (present at 4g/l) into media with (□) and without (■) Chlorella cells present. Growth data for the culture is also included.

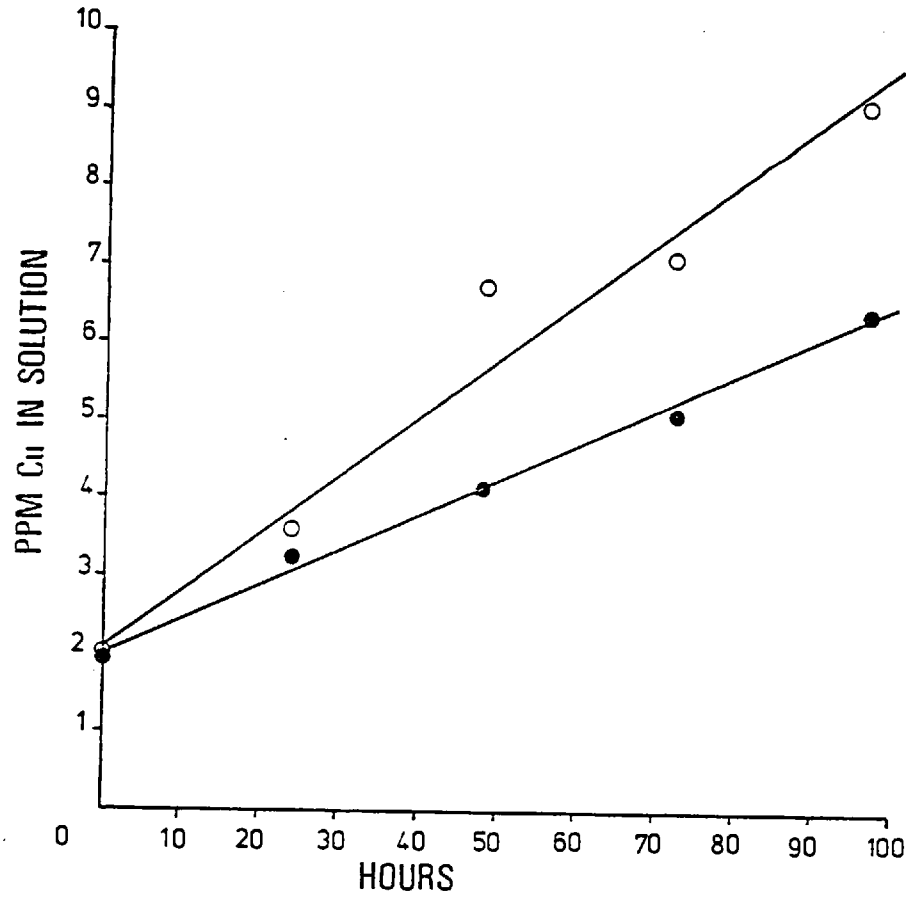
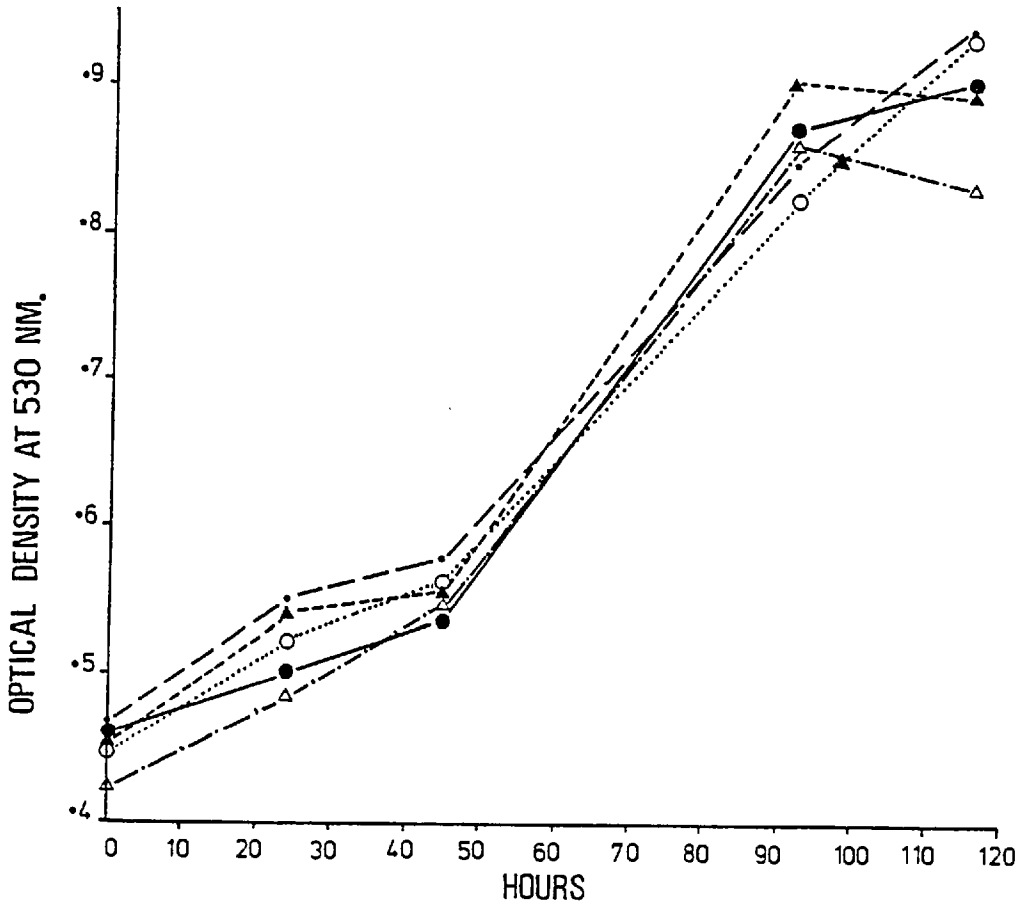


Figure 12. Copper release from Glass B240177 (present at 2g/l) into media with cells present at two different initial densities; 0.01% pcv (○) and 0.09% pcv (●).



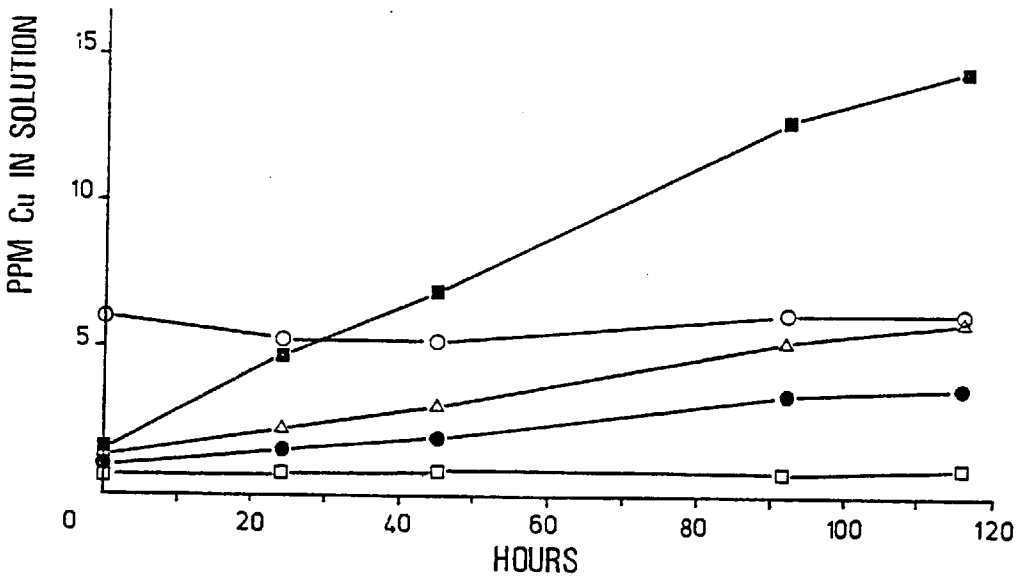


Figure 13. (Top) Growth of Chlorella cultures in media containing various RG additions: control (●),.4g/200mls (•), .8g/200mls (○), 2.4g/200mls (▲) and 5 ppm as the sulphate (△).

Figure 14. (Bottom) Copper levels in solution with various RG additions: Control (□), .4g/200mls (●), .8g/200mls (△), 2.4g/200mls (■) and 5 ppm as the sulphate (○).

between the various treatments. As expected, the 2.4 g RG 200 mls of medium treatment gave the highest copper levels, with a concentration rising to 14.66 ppm. The 0.8 g/200 ml treatment had a final Cu concentration of 6.00 ppm and the other treatments proportionately less, with the 5 ppm copper sulphate varying between 5 and 6 ppm. At the end of the experiment, the cells were digested and figure 15 plots µg Cu per g of cell fresh weight (ppm) against the mean medium copper level over the period of the experiment. For the RG-treated cells, a very good straight-line relationship emerged, although there was statistical overlap between the middle two points. The maximum Cu-content in the cells was, as expected, found in the 2.4 g RG/200 ml treatment where 2507 µg Cu/g cell fresh weight were recorded. A point of note was that a relatively low $\bar{x}$ level of 247 ppm Cu was found in the cells treated with 5 ppm Cu, supplied as the sulphate. This value was significantly lower than the metal levels in cells from the 0.4 and 0.8 g RG/200 ml treatments, which both gave $\bar{x}$ copper levels in the medium less than the 5.84 ppm measured for the sulphate. This therefore seemed to indicate a difference in the uptake of metal from the two types of application.

In order to try and gain a more definite Chlorella growth response to a RG treatment, the above experiment was repeated, but using a less favourable set of growth conditions. Thus, a lower initial cell inoculum (0.015% pcv instead of 0.050% as used above) was employed and no aeration system was included; the flasks were sealed with cling-film. Table VI summarises the data for culture development.

TABLE VI Growth of Chlorella cultures maintained in various RG regimes. (Results are the mean of 5 replicates with 95% confidence limits. *+ signifies that results are significantly greater, and * - signigicantly smaller than control values).

$\bar{x}$ Cu level (ppm) (average of initial + final samples)	Treatment		Optical density (530 nm) at following sampling times (hours). 0	19	25	43	67	91	167
0.95	Controls	$\bar{x}$	.17	.21	.23	.25	.34	.43	.47
		CL	.02	.04	.02	.04	.03	.03	.03
3.41	0.4 g RG/ 200 mls	$\bar{x}$	.23	.25	.25	.32	.39	.45	.52
		CL	.04	.02	.04	.03	.03	.04	.06
5.60	0.8 g RG/ 200 mls	$\bar{x}$	.20	.28	*+ .30	.32	*+ .41	.47	.52
		CL	.02	.04	.02	.03	.03	.03	.05
20.43	2.4 g RG/ 200 mls	$\bar{x}$	.19	*+ .35	*+ .32	.30	.34	*- .36	*- .36
		CL	.01	.03	.02	.03	.02	.02	.03

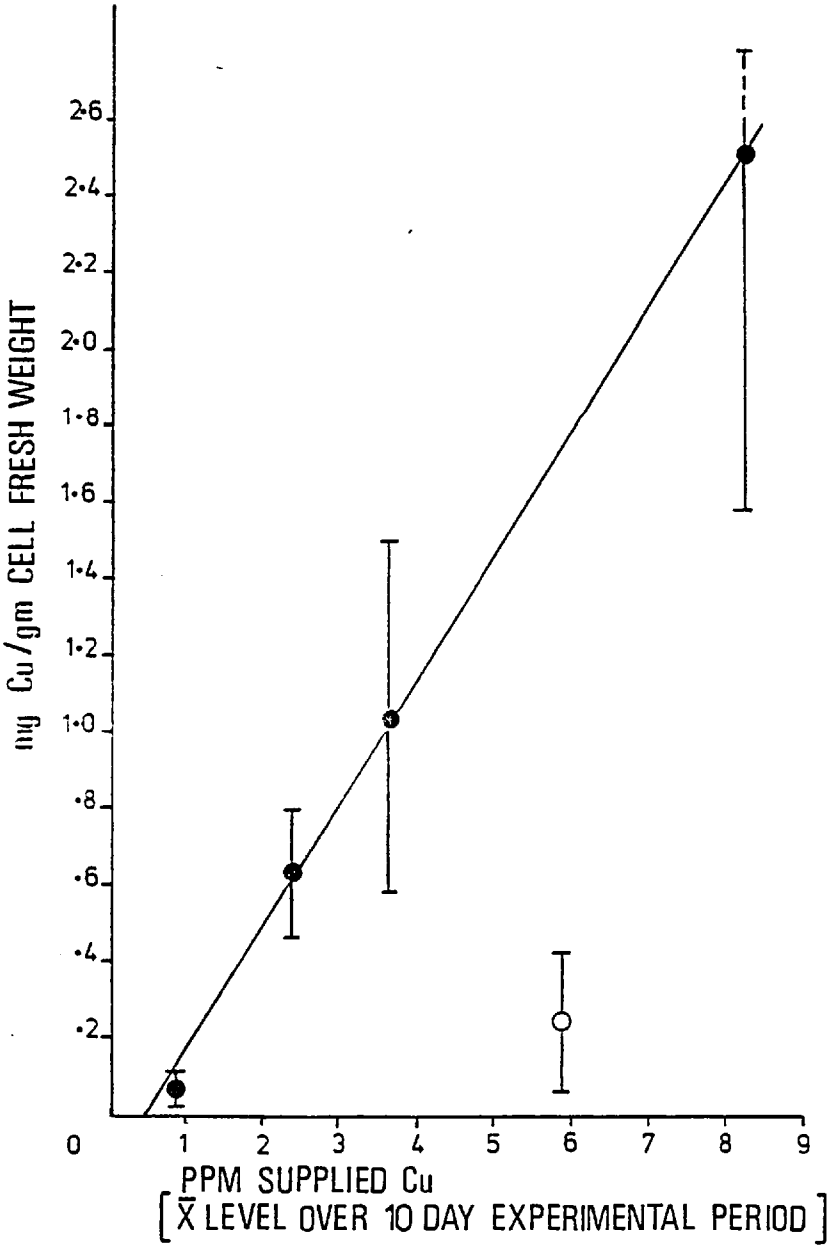


Figure 15. Copper content of Chlorella cells after growth under various copper regimes. (points are the mean of 5 replicates ± 95% confidence limits). ● = copper added as RG, O = copper added as the sulphate.

Again, the effects of the RG on cell growth were not dramatic, although a growth supression could be seen to be developing in those cultures with the highest metal levels. This inhibition was significant, with respect to controls, at the last two sampling times.

These two initial experiments had therefore failed to demonstrate a dramatic growth-retarding effect of RG-supplied copper on algae. Only when a yet higher range of concentrations was used was the effect more definite. Figure 16 gives the growth data from a series of treatments in which up to 3.5 g of RG were used per 200 ml volume. The results show a graded effect of increasing release glass levels. At the highest dose rate (3.5 g RG/200 mls), the results were significantly different from controls at all points except the time zero sample. The copper levels achieved in the media were lower than in the last experiment probably because of the fact that more cells were present (initial inoculum of 0.052% pcv) to take up the metal from solution.

These experiments had therefore basically demonstrated that, although the release glasses could give rise to quite high levels of copper in the media, they could only seriously inhibit culture development when the level of their application was relatively high.

A final Chlorella growth experiment looked at release glass effectivity as a growth retardant under differing complexing situations. Cells with an initial pcv of 0.019% were inoculated into flasks containing 1.5 g RG/200 mls, but with EDTA present at 0.27, 0.10 or 0.01 mM. There were four replicates and seperate sets of controls for each treatment. The controls with less than the normal level of EDTA (0.27 mM) did not, on average, lag behind the true controls by more than 10%. All three sets of controls were therefore pooled and plotted, along with the Cu-treated traces, as figure 17.

The graph demonstrates a clear progression whereby the copper exhibited an increasingly higher degree of growth supression as the chelator level fell. Very severe growth inhibition was particularly noted in the 0.01 mM EDTA treatment, this being a parallel result to that obtained when the metal was added as the sulphate.

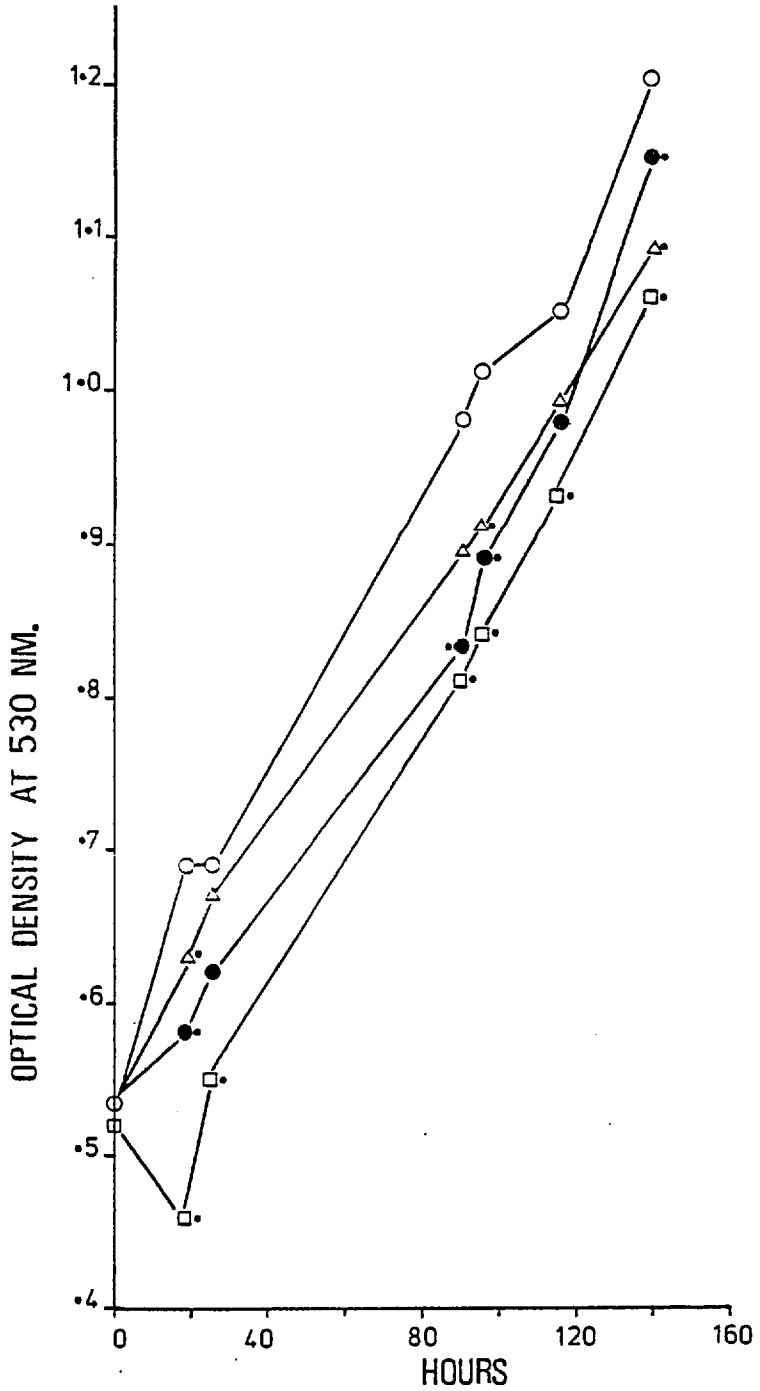


Figure 16. Growth of Chlorella cultures in media containing various RG additions. Control (mean of initial and final copper levels was 0.49 ppm) (○), 1.6g RG/200mls (3.87 ppm mean copper) (△), 2.4g/200mls (4.35 ppm Cu) (●) and 3.5g/200mls (6.21 ppm Cu) (□). (· denotes a significant difference from controls).

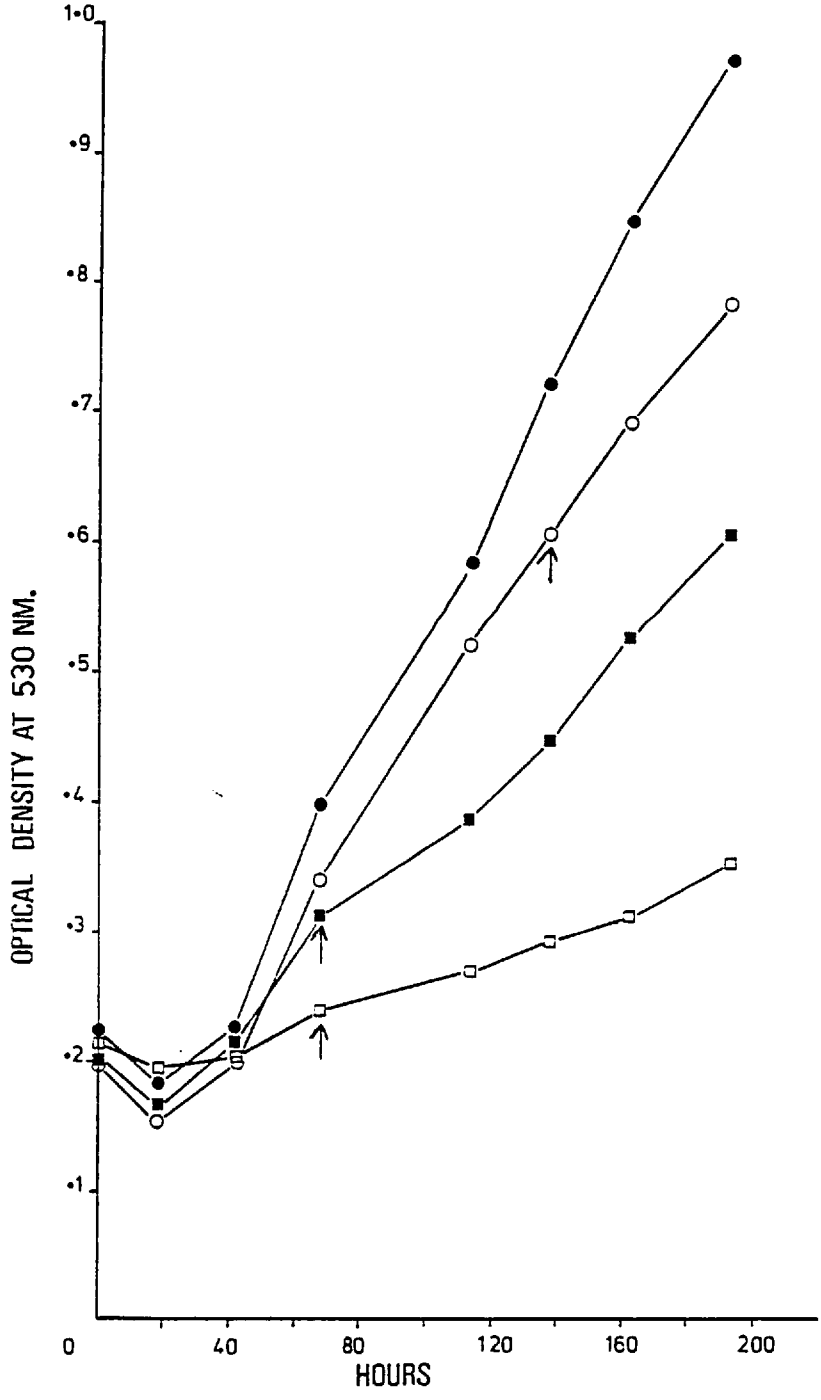


Figure 17. Growth of Chlorella cultures in media containing various EDTA levels and 1.5g RG/200mls. Mean of all 4 sets of controls (0.27 mM EDTA, mean of initial and final copper levels =.036 ppm) (•), 0.27 mM EDTA with RG, mean copper level of 4.83 ppm (○), 0.10 mM EDTA with RG, 3.70 ppm mean Cu (■) and 0.01 mM EDTA with RG, 4.80 ppm mean Cu (□). ↑ denotes the point at which the results become significantly, and thereafter consistently, different from controls.

3.1c Copper uptake experiments.

(i) Chlorella's effects on medium pH.

This experiment was conducted so that an idea could be gained of Chlorella's effects on medium pH; the pH is of relevance to the solubility and availability of copper in solution. Table VII gives the pH values monitored in the various media over the experimental period.

TABLE VII Time-course monitoring of medium pH with and without a Chlorella inoculum (0.1% pcv), aeration and stirring.

Treatment		pH values at the following sampling times (minutes)						
		5	30	60	130	200	240	300
Aerated + stirred	with Chlorella	6.370	6.370	6.345	6.420	6.515	6.565	6.600
No aeration or stirring	cells	6.400	6.440	6.445	6.555	6.650	6.680	6.685
Aerated + stirred	No cells	6.380	6.360	6.350	6.340	6.350	6.350	6.320
No aeration or stirring	present	6.385	6.380	6.370	6.385	6.380	6.385	6.365

The results clearly show that the presence of Chlorella cells results in a raising of pH values, but that the effect is decreased by aeration and stirring.

(ii) Indirect monitoring of copper uptake.

The rise in medium pH brought about by the cells may have been causing increased precipitation of metal. These experiments were therefore run so that the uptake of metal could be monitored indirectly by reference to remaining levels in solution, the loss of dissolved copper being engineered to be the same in inoculated and cell-free samples.

The results from five such experiments, each undertaken with slightly different conditions, are plotted as figure 18. The results were disappointing in that replicate variability was so great that statistically-significant differences between copper levels in inoculated and cell-free media were infrequent. In the two traces without EDTA present, two contradictory trends emerged whereby in one case (with the higher copper levels), more metal was in solution when cells were present while, in the other, the reverse was true. When EDTA was included in the medium, there was a trend in all three experiments for copper levels to be slightly higher in the cell-free

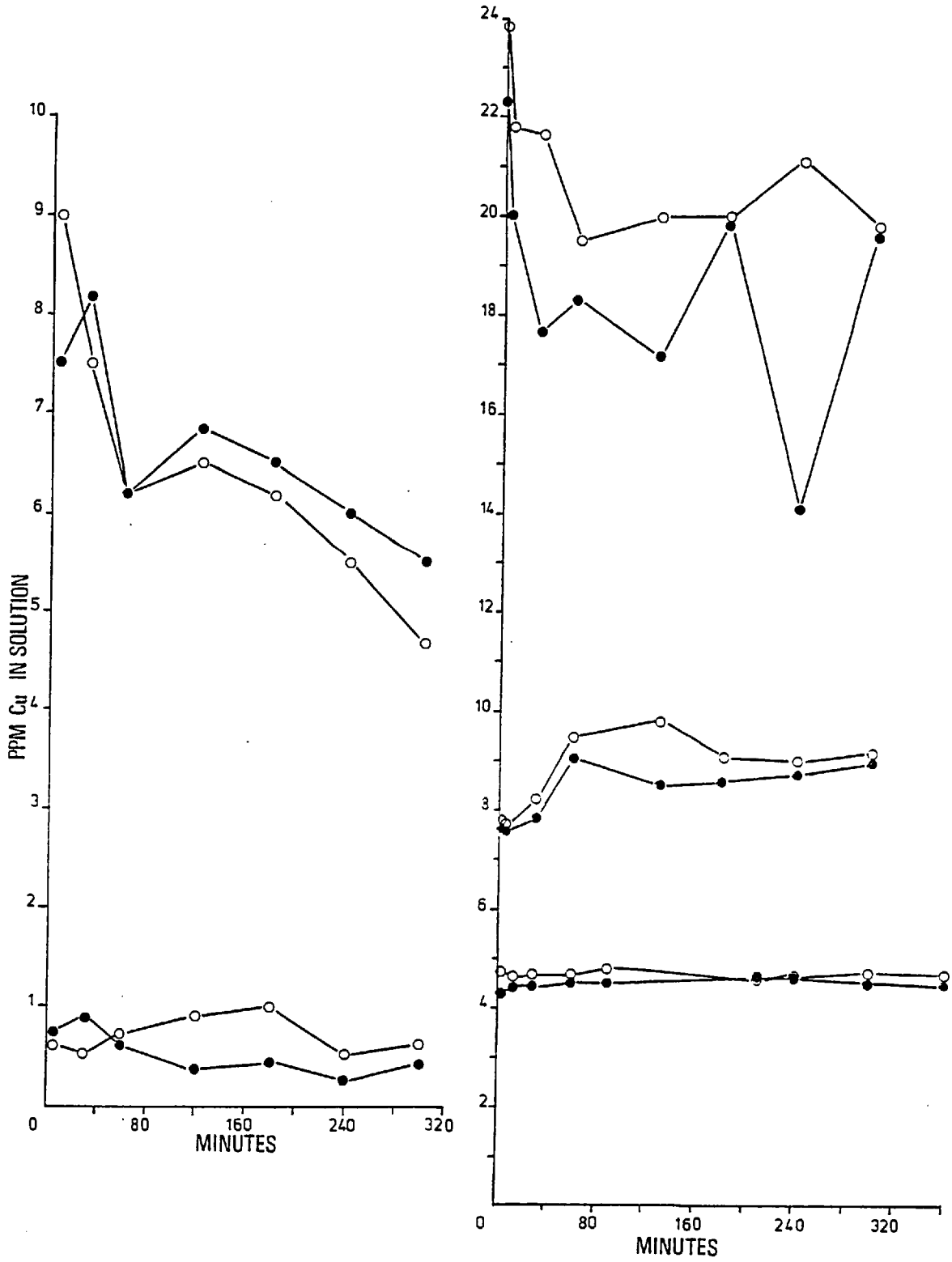


Figure 18. Changes of copper levels over time in solution with (•) and without (o) Chlorella cells and EDTA present. The traces on the left were in the absence of EDTA, while it was present for those on the right. The bottom pair of traces on the right were produced with no micronutrients present.

media. The results depicted as the bottom right pair of traces were gained from an experiment with no micronutrients present in the medium. It had been expected that this might have led to increased copper uptake by the cells, but the two traces were virtually identical.

These experiments have therefore failed to clearly demonstrate copper uptake by the cells under the conditions employed. The method was partially unsatisfactory because it was indirect. A slight tendency for metal levels in inoculated media to be less than in cell-free samples was indicated, but the variability in the results left this unproven. It may be that, with the level of cells used and the relatively-short experimental periods employed, it was unrealistic to expect significant changes in the levels of the bulk solution.

(iii) Direct monitoring of copper uptake.

The digestion of samples of standard Bowen's Kale showed that, on average, the digestion technique yielded results that were accurate to within 8% of the true total of metal present.

An experiment was conducted to monitor the various levels of copper in a Chlorella culture over time. Readings of total copper, medium copper (in solution) and copper in the cells were obtained. The traces given in the graphs comprising figure 19 are for the three seperate treatments used in the experiment ($\bar{x}$ total copper levels of 1.77, 4.38 and 18.24 ppm, depicted as ● , o and ▲ respectively). In this figure, graph A shows total copper levels in the cultures over time. The graph points were based on single results and are consequently rather variable. However, the graph serves to illustrate that the total copper levels in the three treatments were consistently distinct over the experimental period. Graph B shows the levels of copper in the medium. All three traces fall with time, the decline being greatest in the highest copper treatment. Graph C draws the mean copper levels within the Chlorella cells, maintained in the various treatments over the experimental period, against the $\bar{x}$ total copper in the culture. Here, a good straight line relationship was demonstrated, indicating that the more total copper present, the more will be taken up by the cells. Graph D summarises the results of the growth monitoring carried out during the experiment. There was increasingly-severe growth inhibition as the copper level rose.

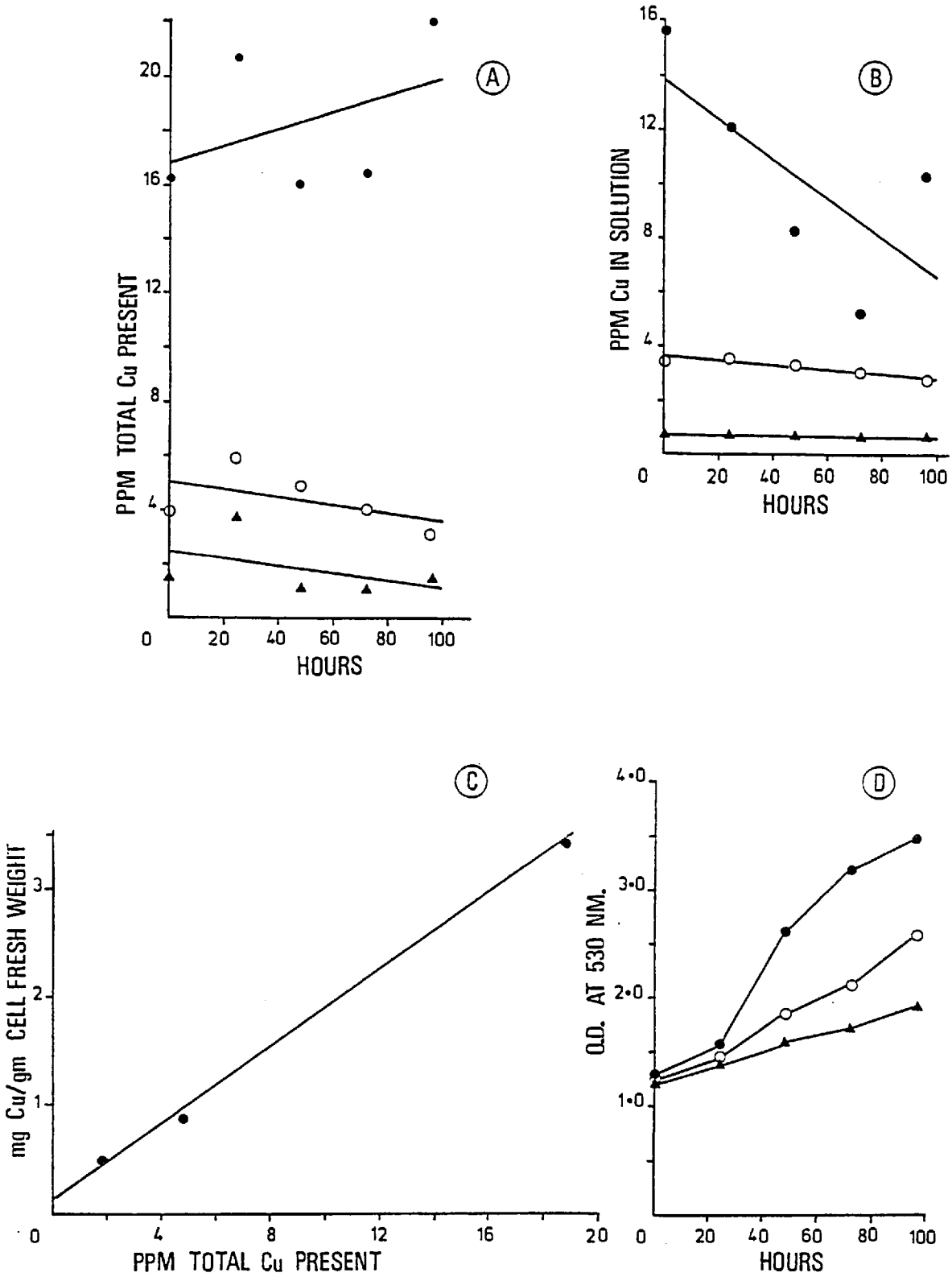


Figure 19. The monitoring of Total (graph A), Medium (graphB) and cellular (graph C) Cu levels in a Chlorella culture, the metal being supplied as the sulphate. Graph D shows culture growth over the experimental period (▲ denotes a mean total copper concentration of 1.77 ppm, ○ of 4.38 ppm and • of 18.24 ppm).

This type of overall copper-monitoring experiment was repeated using Release Glass at 0.8 g l^{-1} as the copper source. Figure 20 gives the results obtained. Graph 1 of this figure, summarising total copper levels in cell-containing and cell-free media with and without release glass present, show that total copper levels in the normal culture were more or less steady, but that they rose with time, as expected, when a release glass was included. The same was true for media copper levels over the experimental period. There was a trend for the copper content of the cells themselves to increase with time; this increase was more marked in the case of the culture containing RG, but there was also a slight time-dependant rise in the normal culture. The development of the cultures was roughly monitored by noting the fresh weight of cells from culture aliquots (same aliquots as used for cell-digestions). The traces nonetheless indicate that there was a severe growth inhibition in the RG-containing media.

(iv) Uptake of copper by live and scalded cells.

Figure 21 summarises the data from two experiments in which copper uptake by live and scalded cells was followed over time, but there were different initial pcv (0.08% and 0.28% graph 1 and 2 respectively). The result were probably variable because of the small weights of cells that were involved and which were difficult to assess accurately. However, a trend was visible whereby scalded cells contained more copper than did normal cells; on only two occasions out of 13 sampling times were the copper content values for normal cells greater than for scalded cells. It was also demonstrated that there was more copper present in the cells (both treatments) of the first experiment with the lower initial pcv than in the second with a higher initial cell density.

3.1d Work with an oxygen electrode.

Figure 22 shows the effects of a range of copper concentrations from 5 to 50 ppm on Chlorella photosynthesis, respiration and growth. The results are plotted as a percentage of appropriate control rates. It can be seen that, under the conditions used, 30 ppm copper and above completely inhibited photosynthesis within a few minutes. The trend in copper treatments at less than 30 ppm was less clear; there seemed to be little difference in the responses of the cells to treatments at 5ppm or 20 ppm. A threshold effect may have been operating or the biological variability of the material may have minimised the differences.

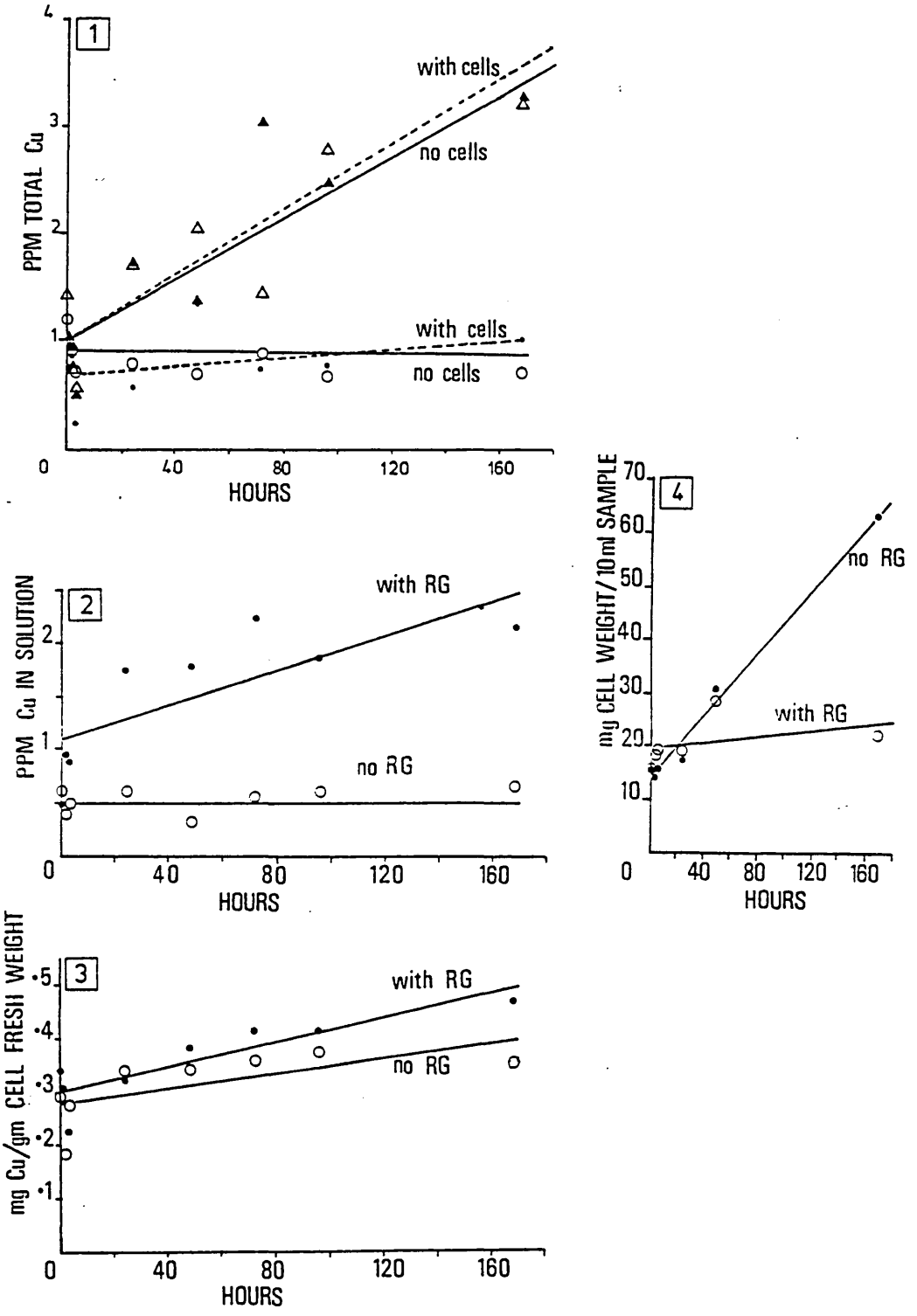


Figure 20. The monitoring of Total (graph 1), Medium (graph 2) and cellular (graph 3) copper levels in a Chlorella culture over time, the metal being supplied as a RG. Graph 4 shows culture growth over the experimental period. (In graph 1, △ and ▲ denote plus RG, while ○ and · denote no RG present).

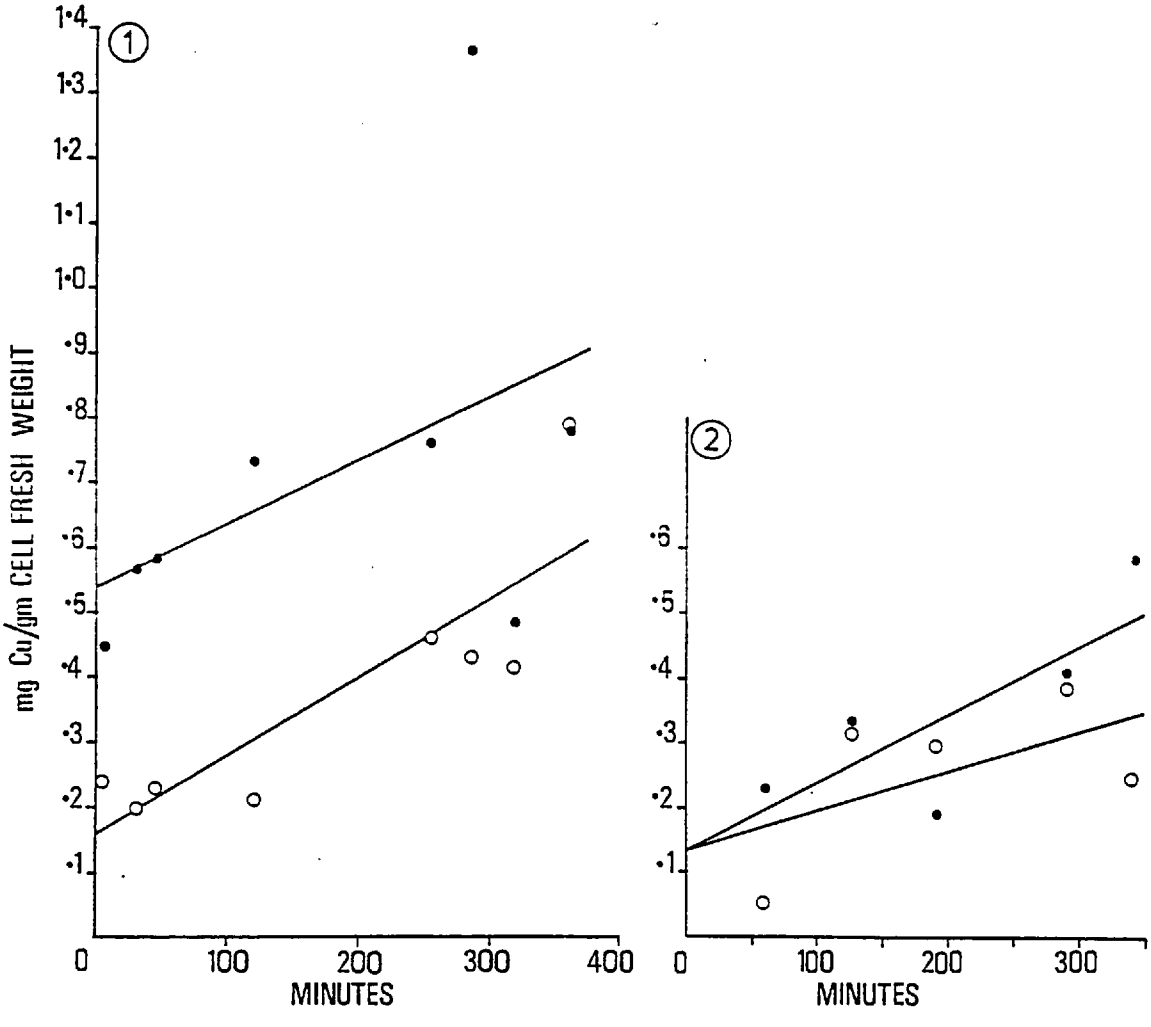


Figure 21. Copper uptake by normal (o) and scalded (•) cells over time. (Graph 1 had an initial inoculum of 0.08% and graph 2 of 0.28%).

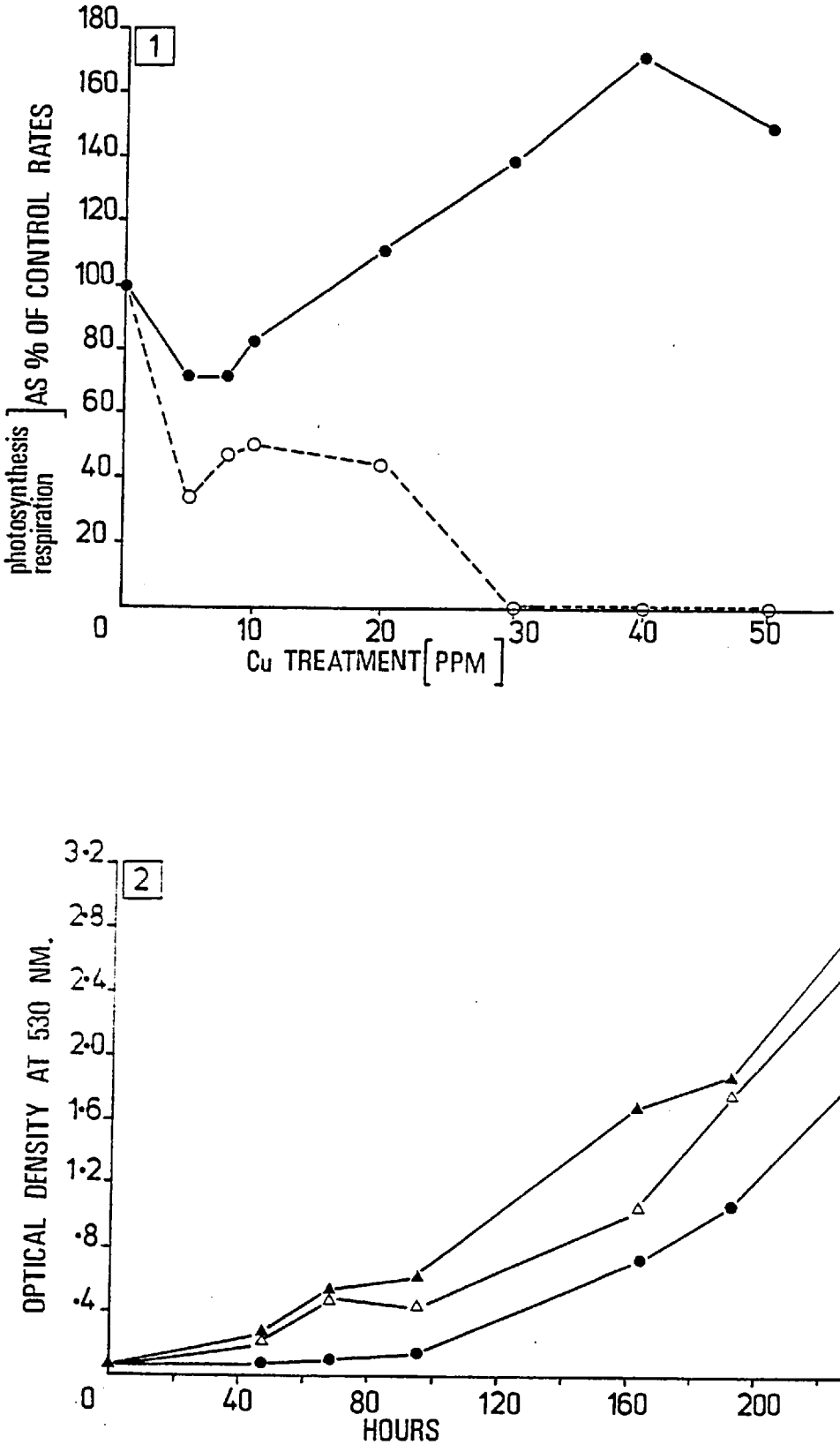


Figure 22. Graph 1, Effects of copper treatments on photosynthetic (○) and respiratory (●) rates in Chlorella cells, each being expressed as a % of control rates. Graph 2, Growth of Chlorella cultures, the inocula deriving from cells used in the above oxygen electrode studies. (▲, 0 ppm Cu treatment in O_2 electrode, △ 5 ppm treatment and • 50 ppm Cu treatment).

A different picture emerged from the respitation graph trace. This showed a smallish inhibition at the lower copper treatments to 83% of controls at 10 ppm), but there was there-after a stimulation of the rate, rising to 172% of controls at 40 ppm. The respiration rate at 50 ppm appeared to indicate a further downward trend, i.e. for the copper to once again become increasingly inhibitory.

Samples of cells from some of the above electrode treatments were used as inocula for cultures in normal fresh media. The results of the growth monitoring are presented as graph 2, figure 22. Under the circumstances used, it can be seen that the copper treatments, although only acting on the cells for around 15 minutes, seem to have had quite a retarding effect on culture development. The inhibition, with respect to controls, became less marked with time. The differences in growth appeared greater than in previously-reported time-of-exposure to copper experiments.

3.1e Copper's effects on chlorophyll levels and a:b ratios.

Table VIII gives the data for various experiments in which the effects of a metal treatment on total chlorophyll levels within Chlorella cells were investigated.

TABLE VII Total chlorophyll content of Cu-treated cells as percentage of controls.

Treatment (ppm Cu)	Initial pcv of cells (%)	Time (hours) 0-.1	1	2	4	6	24	28	48	overall $\bar{x}$
3.45	0.053	103	94	96	104	-	-	-	-	99
9.60	0.160	74	108	115	107	97	-	-	-	100
18.10	0.160	91	74	82	57	53	36	22	21	55

The results show that niether 3.45 nor 9.60 ppm copper had any real significant effect on total chlorophyll levels over the experimental period. The results for 18.10 ppm, however, showed a definite decline in levels with time, the effect being apparent from the first few hours.

Figure 23 displays the results of a series of experiments that looked at copper's effects on chlorophyll a:b ratios in Chlorella. With respect to controls, 9.60 ppm had no effect on a:b ratios, while 18.10 and 21.00 ppm caused significant decreases

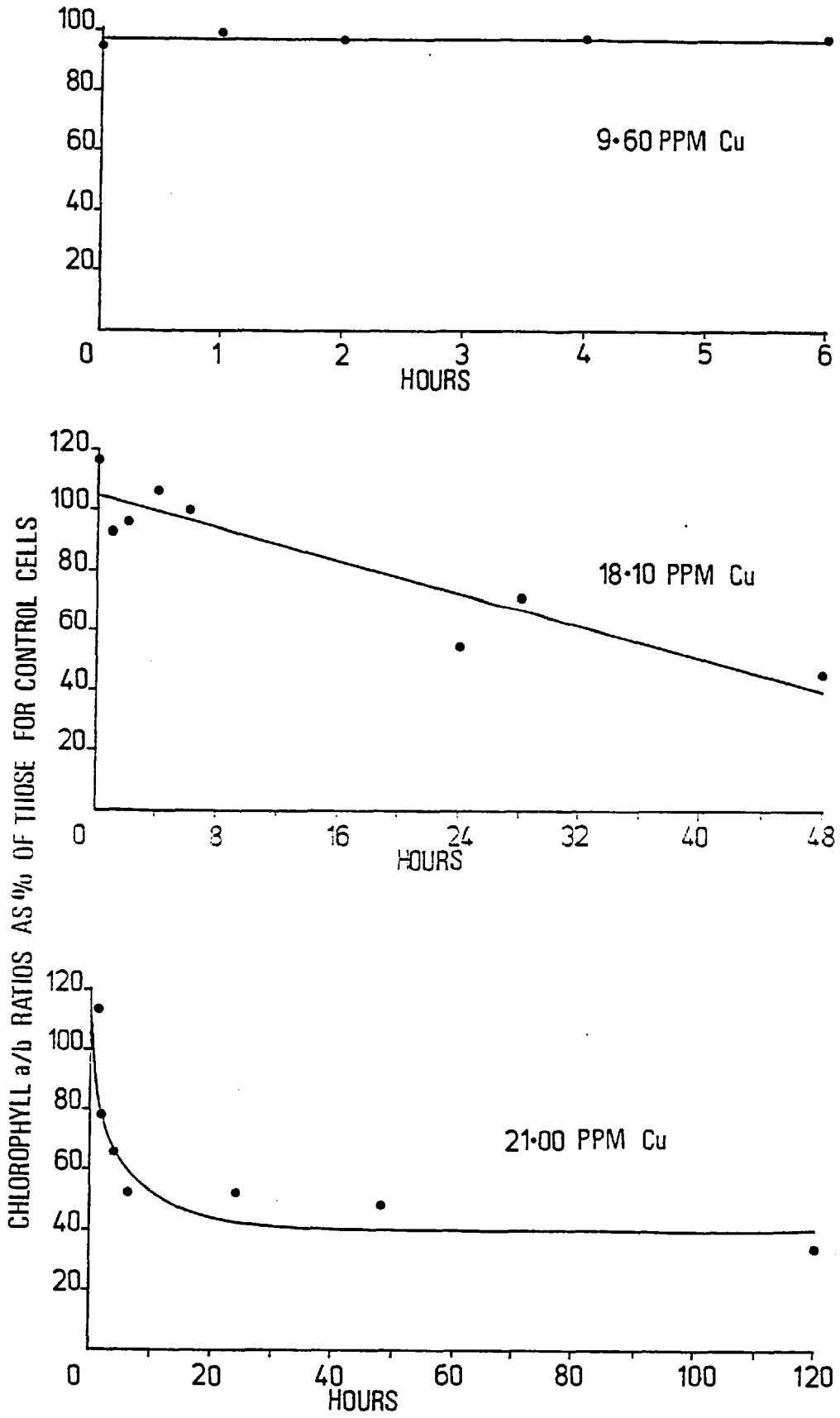


Figure 23. Changes in the chlorophyll a:b ratios of Chlorella cells maintained in various copper treatments. Results are expressed as a % of control ratios.

in the values. This indicates a fall in the level of chlorophyll a pigment present. The 18.10 ppm trace appeared to be a straight line, whereas that for 21.00 ppm took more the shape of a curve; the reason for this difference was unclear, although it must be remembered that the time scales for the two traces are different.

Table IX depicts total and chlorophyll a:b ratio results from an experiment using 19.63 ppm copper and in which both aerobic and anaerobic conditions were employed.

TABLE IX The effects of copper on total chlorophyll levels and on chlorophyll a:b ratios in Chlorella cells maintained under aerobic and anaerobic conditions. (All results are expressed as a % of appropriate control values).

Treatment (ppm Cu)	Time (hours) 0-.1	4	7	24	28	
19.63 Aerobic	79	42	49	41	42	Total Chlorophyll
Anaerobic	87	49	57	42	48	
19.63 Aerobic	98	77	69	24	27	Chlorophyll a:b Ratios
Anaerobic	101	83	64	27	25	

From these results, it can be seen that, although declines in both total chlorophyll and a:b ratios are again evident over time, no real difference has been demonstrated between aerobic and anaerobic cultures.

3.2 Work with the phaeophyte Sargassum muticum.

3.2a Growth experiments.

The first experiment aimed to determine the conditions under which this alga could best be grown. With other conditions as detailed in the materials and methods section, growth was compared under light regimes of 8 and 16 hours daily illumination. Graph 1, figure 24, givesthe two graph traces for 5 cm lateral segments over 52 day period. This preliminary work clearly demonstrated a much higher growth rate with the longer photoperiod, and therefore 16 hours daily light were used in all experiments thereafter.

This pre-run was followed by a normal growth experiment, using a range of

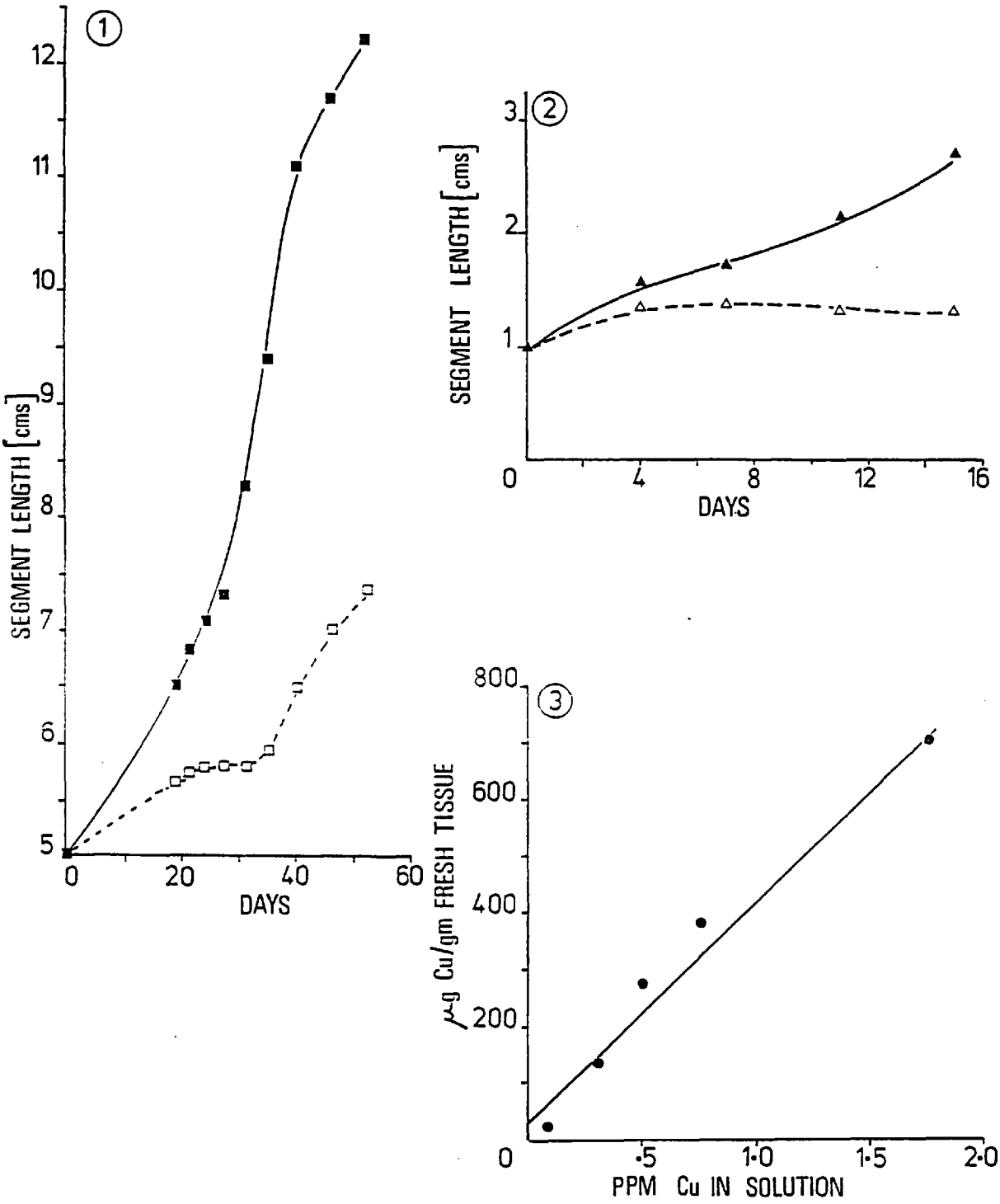


Figure 24. Sargassum muticum: Graph 1, Growth of 5 cm Sargassum segments under 16 hours (■) and 8 hours (□) daily illumination. Graph 2, Growth of 1 cm Sargassum segments under control (▲) and 0.31 ppm Cu (△) conditions. Traces for 0.49, 0.76 and 1.75 ppm were essentially the same as for 0.31 ppm and have therefore been omitted for clarity. Graph 3, Copper content of Sargassum tissues after 15 hours in various copper treatments.

low copper concentrations (as analysed) up to 1.75 ppm. The results are drawn as graph 2, figure 24. Only the control and 0.31 ppm traces are included as the other metal treatments (0.49, 0.76 and 1.75 ppm) all gave nearly-identical results. It can be seen that all of the copper treatments completely inhibited Sargassum growth. Each point was the mean of 30 replicates and except for one result, all treated tissues were significantly smaller (95% confidence limits) than controls at all sampling times except time zero. At the end of the above experimental period (15 days), the algal tissues were digested and analysed for copper content. The results are drawn as graph 3, figure 24. A very good straight line relationship was demonstrated between copper in the tissues and copper in solution.

3.2b Survival experiments using respiration as a criterion for assay.

The results of the first of these experiments is summarised in graph 1, figure 25. This figure demonstrates a dramatic decrease in the proportion of the tissues respiring at plus four days, this being caused by only a relatively small increase in copper levels. An LD_{50} of 0.22 ppm copper was read off from the graph .

The experiment was then repeated using RG B240177 as the copper source. This time, the segments were harvested at plus 3 days and were then analysed for copper content. Graph 2, figure 25 summarises the data obtained. Again, a rapid decrease in the number of respiring tissues was found to be associated with only a small increase in copper levels; an LD_{50} of 0.24 ppm copper was found. An increase in the copper content of the tissues was found with increasing external copper. The graph is a different shape to that obtained in graph 3, figure 24, largely because of the different scale used.

3.3 Work with isolated pea chloroplasts.

3.3a Electron transport work.

(i) Effects of copper on overall electron transport to methyl viologen.

Methyl viologen becomes oxidised at a site after PS I as a result of electron flow through the complete photosynthetic electron transport system from water. The point of methyl viologen oxidation is shown in figure 26 below.

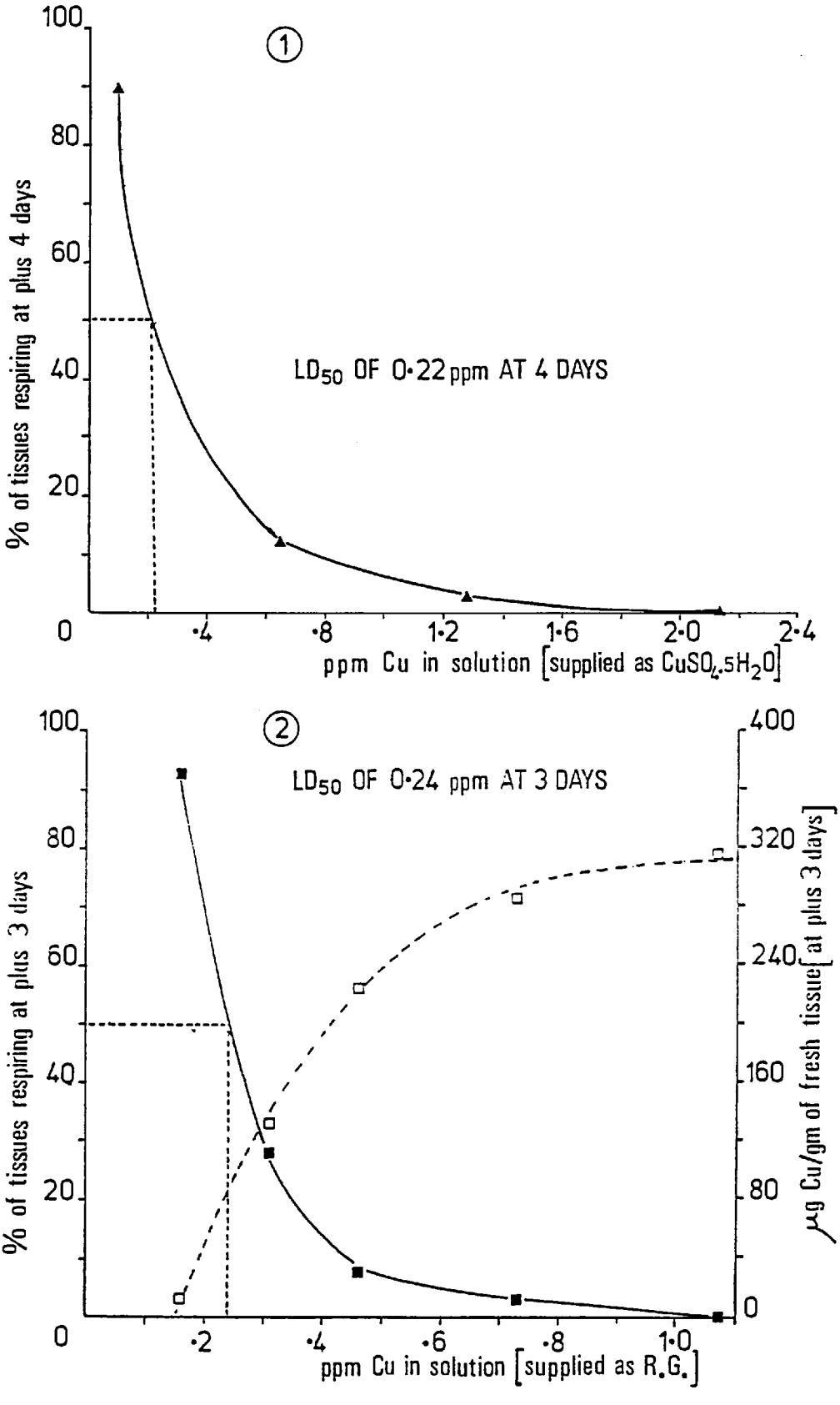
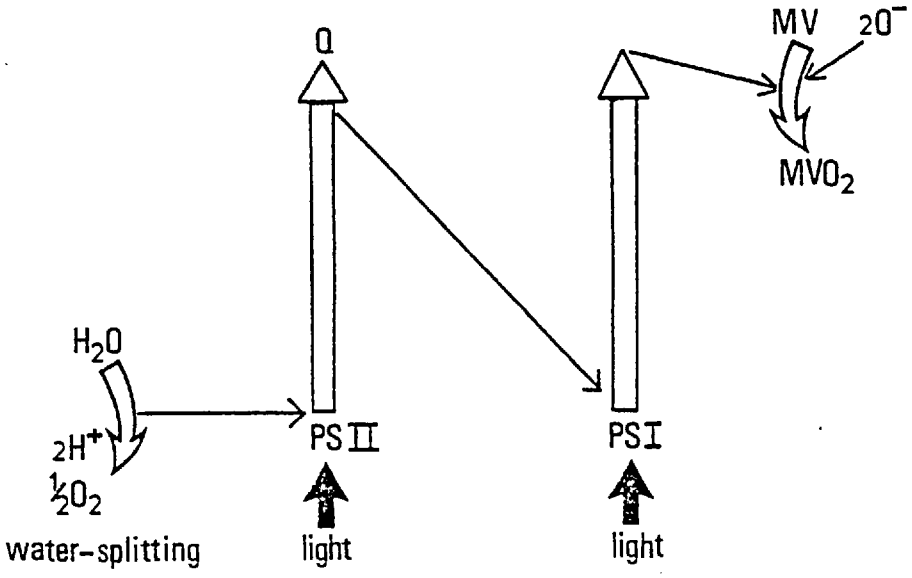


Figure 25. Graph 1, % of Sargassum tissues respiring at plus 4 days in various copper treatments, the metal being supplied as the sulphate. Graph 2, % of tissues respiring at plus 3 days in various copper treatments, the metal being supplied as a RG (■). Copper content of the various tissues at the end of this period is given as (□).

Figure 26. Outline of the system employed when assessing overall electron flow to MV.



The nett result of electron flow through this system is oxygen uptake; one oxygen atom from water and one from solution is utilised per MV oxidation event. Activity of the system is therefore monitored by reference to the rate of oxygen uptake from the reaction medium.

As most of the electron transport studies conducted utilised copper sulphate as the metal source, a preliminary experiment investigated the effects of sulphate ion (SO_4^{2-}) alone on electron flow to methyl viologen. A 15.74 mM K_2SO_4 standard was employed in these studies, this containing the same amount of sulphate ion as does a 1000 ppm copper standard. Table X details the amounts of this standard used for particular treatments.

TABLE X Levels of SO_4^{2-} ion employed in experiments with methyl viologen.

Vol. 15.74 mM K_2SO_4 used in 2 mls (µl)	K_2SO_4 conc. in reaction mixture (µM)	Copper level containing identical SO_4^{2-} level (ppm)
2	16	1
4	31	2
8	63	4
10	79	5
16	126	8
20	157	10
30	236	15
60	472	30
120	944	60

Graphs 1 and 2 of figure 27 show the effects of a range of K_2SO_4 concentrations on coupled and uncoupled rates of electron transport respectively. The highest K_2SO_4 concentration used gave a SO_4^{2-} concentration equivalent to that present in a 60 ppm copper treatment. This range of concentrations gave rise to a reduction in O_2 uptake rates, with respect to controls, but even at the highest level of application, the coupled and uncoupled rates were still 66 and 79% of controls respectively (and this concentration is twice as high as that used in any other experiment). The reductions in rates gained using lower levels were proportionately smaller. Uncoupled rates appeared less sensitive to SO_4^{2-} inhibition than did coupled. Graph 3, also included in figure 27, shows the results of work in which rates of coupled and uncoupled electron transport to methyl viologen were assessed after various times of preincubation, up to 20 minutes, in 472 μM K_2SO_4 (equivalent SO_4^{2-} level to that with a 30 ppm copper treatment). It can be seen that, with this level, uncoupled rates were stimulated slightly while coupled rates were inhibited a little. Expressed as a percentage of controls, the mean value for O_2 uptake rates over the five sampling times for coupled and uncoupled rates were 91 and 111% respectively. These initial experiments therefore gave an idea of the magnitude of the effects of SO_4^{2-} alone on electron transport rates.

The effects of a range of copper concentrations on electron flow to methyl viologen are shown as graph 1, figure 28. Both coupled and uncoupled rates are shown. Absolute control rates were typically around 60 μM O_2/mg chlorophyll/hour for coupled chloroplasts, with a 3 to 5 fold increase after uncoupling. The traces show that uncoupled rates of electron transport fell with increasing copper levels, but there seemed to be a trend for the highest metal treatments to give an apparent increase in coupled rates. This was further investigated in later experiments; it almost seemed as if copper was in some way acting as an uncoupler in the higher concentration range.

A further experiment, summarised as graphs 2 and 3 figure 28, looked at the effects of various times of preincubation in four different copper levels. The traces, for both coupled and uncoupled rates, show a graded response to increasing copper levels, but they also demonstrated that most of the effect of any given copper treatment was apparent very early on and that there was, thereafter, relatively little further decrease in rates. In other words, a very high proportion

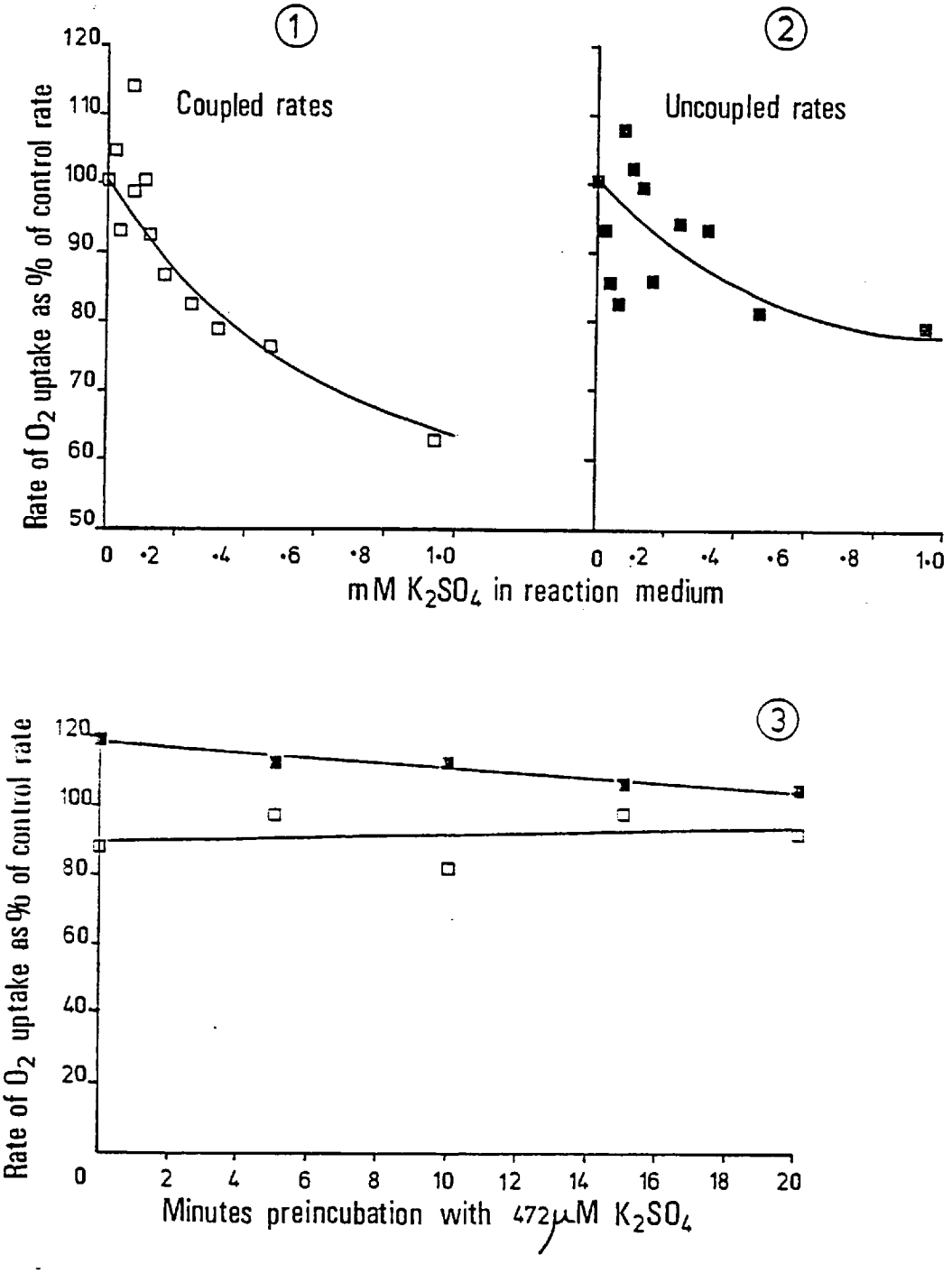


Figure 27. Effects of SO_4^{2-} ion on coupled (□) and uncoupled (■) rates of electron flow to methyl viologen. Graph 1 and 2 show the effects of various K_2SO_4 concentrations, and graph 3 investigates the effects of exposure to 472 μM K_2SO_4

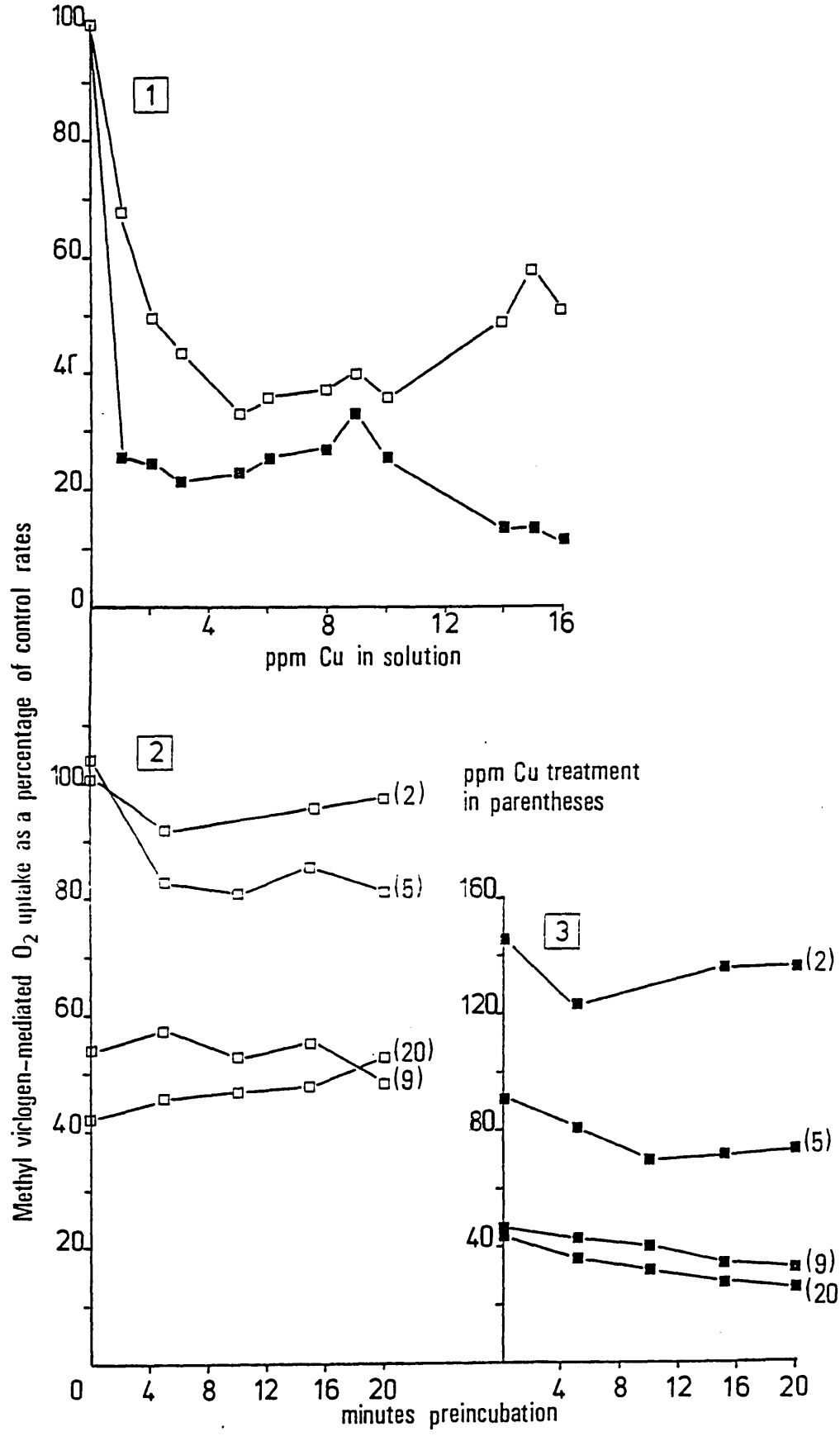


Figure 28. Effects of various copper treatments, and times of preincubation, on rates of MV-mediated O_2 uptake (□ = coupled rates, ■ = uncoupled rates).

of the chloroplast response to the metal treatment was usually evident by the time zero sample. The 20 ppm coupled trace again seemed to show a slight increase in rate over time. The same trend was again visible for uncoupled rates to be more severely affected than coupled.

A series of experiments, summarised as Table XI, was then conducted to investigate any differences between the effects of copper treatments on MV-mediated O_2 uptake when the preincubation was in the light and the dark.

TABLE XI Light versus dark preincubation; copper effects on MV-mediated O_2 uptake (expressed as % of controls).
(Figure in brackets refer to uncoupled rates).

Minutes of preincubation	Treatment							
	2 ppm Cu		5 ppm Cu		7 ppm Cu		10 ppm Cu	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
0	85 (63)	101 (72)	69 (31)	52 (31)	56 (11)	132 (26)	48 (15)	45 (16)
5	79 (57)	81 (72)	67 (22)	65 (29)	41 (10)	90 (19)	67 (8)	45 (15)
10	110 (54)	99 (70)	84 (18)	75 (28)	47 (8)	83 (20)	66 (6)	54 (13)
15	106 (62)	118 (78)	86 (19)	69 (30)	35 (7)	69 (19)	58 (7)	77 (13)
20	99 (65)	87 (70)	103 (17)	55 (25)	26 (6)	53 (19)	58 (9)	61 (12)

For uncoupled rates, it can be seen that there is a consistent trend for those chloroplasts preincubated in the dark to be less affected by the metal treatment than were those exposed to light. The difference appeared a little less marked as the copper treatment increased in strength. The overall picture appeared slightly less simple for coupled chloroplasts. At 2 ppm, the light and dark results were very similar and probably not at all different. At 5 ppm, the two sets of results are distinct, the dark-treated samples being apparently more inhibited. As there is a trend for the light-treated rates to actually increase, with respect to controls, over the 20 minute preincubation period, the greater inhibition in the dark may have been caused by the apparent uncoupling effect of the metal, previously mentioned suggesting that this uncoupling effect is at least partially light sensitive. In the 7 ppm-treated samples (coupled), the situation was reversed with the dark-treated chloroplasts suffering the least inhibition. In fact, compared to controls, the dark-treated samples were inhibited roughly only half as much as those exposed to light. The light and

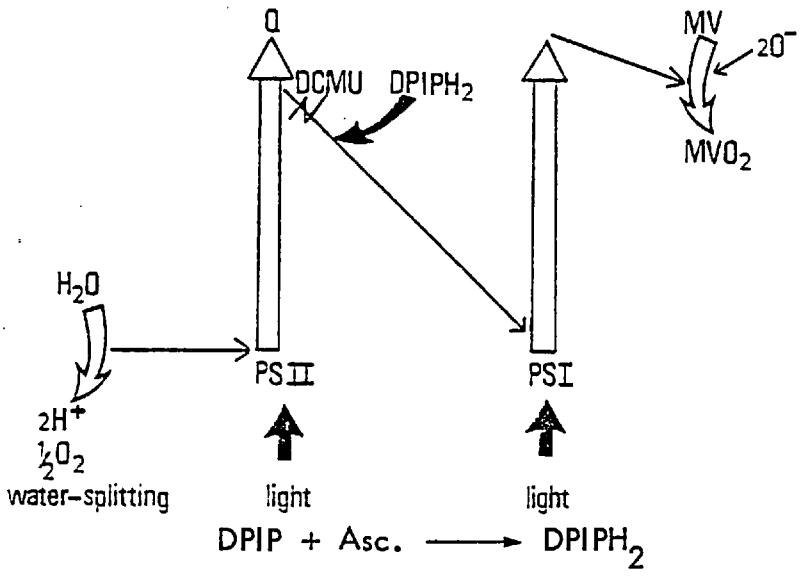
dark data for 10 ppm overlap considerably, probably indicating that the two samples were behaving broadly similarly in response to the metal intrusion. It may be that 10 ppm is simply too high a level for pretreatment in light or dark to make any significant difference to coupled rates.

Overall, it therefore appears that pretreatment in the dark reduces copper's adverse effects in uncoupled chloroplasts over the range 2 to 10 ppm copper, but that, in coupled chloroplasts, the effects is less clear at low concentrations (2 ppm) and also at higher (10 ppm). The overlap of results at 10 ppm may again perhaps be explained by the metal acting as an uncoupling agent.

(ii) Effects of copper on PS I electron transport.

The system employed for photosystem I assessment was as outlined below in figure 29. Dichlorophenol indophenol (DPIP) and ascorbate were the electron-donating combination and methyl viologen was again the acceptor.

Figure 29 Outline of the system employed for PS I assessment.



The oxygen uptake by the ascorbate was strongly catalyzed by copper, and so the chloroplasts were pretreated with metal, seperated out by spinning, and only then were the other reactants added. At high levels of copper treatment, perhaps at 15 ppm but certainly at 20 ppm, sufficient copper was still carried over to the final reaction medium for it to have catalytic effects on the oxygen uptake rates, and so only levels of 10 ppm and below were employed. It appeared that only the

copper of the bulk solution was catalyzing the oxygen uptake by ascorbate. Evidence for this was that the oxygen uptake rate by ascorbate in the dark was slightly less if chloroplasts were present than if they were not. It therefore seemed that copper bound to the thylakoids was not capable of catalyzing the ascorbate reaction.

Figure 30 summarises results from experiments in which copper effects on PS I electron transport were investigated. 1, 3, 5 and 10 ppm treatments were employed and preincubation times up to about half an hour. Results are expressed as a percentage of $\bar{x}$ control rates and coupled and uncoupled data are drawn seperately. Absolute control rates were typically of the order of 10 μM O_2/mg chlorophyll/hour (coupled chloroplasts after the washing procedure). After uncoupling, the rates normally rose to around 70 or 80 μM O_2 uptake/mg chlorophyll/hour. The graphs show that the uncoupled traces follow a neat progression with a decline in PS I O_2 uptake rates with increasing preincubation time, and with the traces for seperate copper treatments being distinct. The picture was again more complex for coupled rates; at some points, there was a stimulation above control levels, this being more noticeable at the two highest copper treatment levels (5 and 10 ppm). It appears that copper is again possibly having two effects which act to confuse the situation; an inhibitory effect on PS I electron transport has been demonstrated, but some sort of stimulation of coupled rates, reminiscent of uncoupling, also seems to be operating. The relative effects of a fixed level of copper application (5ppm, 79 μM) on PS I O_2 uptake in chloroplast suspensions with various chlorophyll concentrations were investigated. This aimed to try and shed more light on the stimlation of coupled rates by copper under certain circumstances.

The data, displayed as Figure 31, shows an apparent stimulation of coupled rates, above control levels, at all the three chlorophyll concentrations used. This stimulation was greatest at the lowest chlorophyll concentration and least at the highest. Again, this could be interpreted as copper in some way causing uncoupling.

In the case of uncoupled rates, there was less inhibition of O_2 uptake in the 100 μg chlorophyll per ml treatment than at 25 or 50 μg chlorophyll which followed a more similar pattern. This result is probably just a dilution effects with more copper being available per thylakoid when the chlorophyll concentration was low.

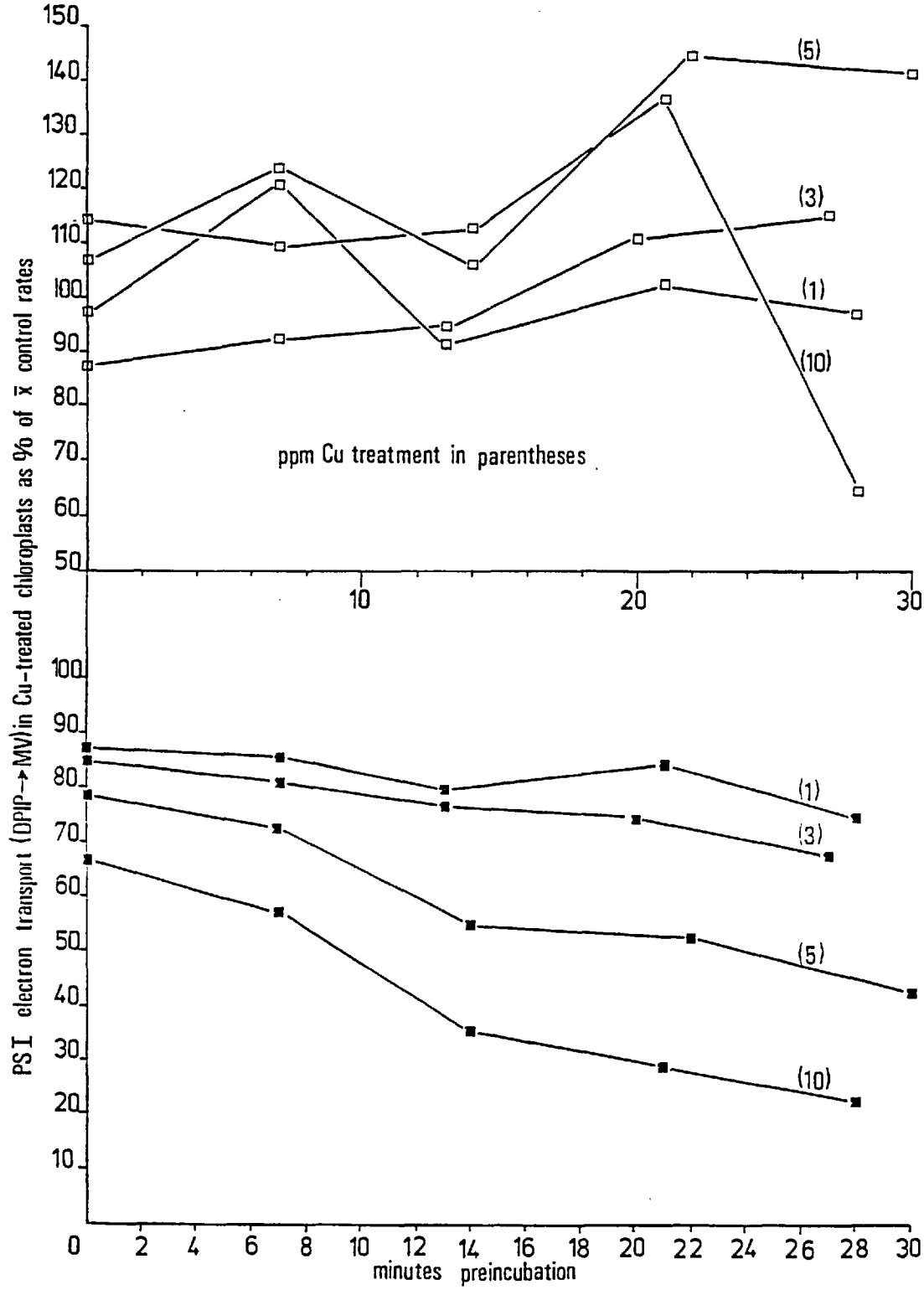


Figure 30. Effects of 4 different Cu treatmentson coupled (□) and uncoupled (■) rates of PS I electron transport to MV. Results are expressedas a % of mean control rates.

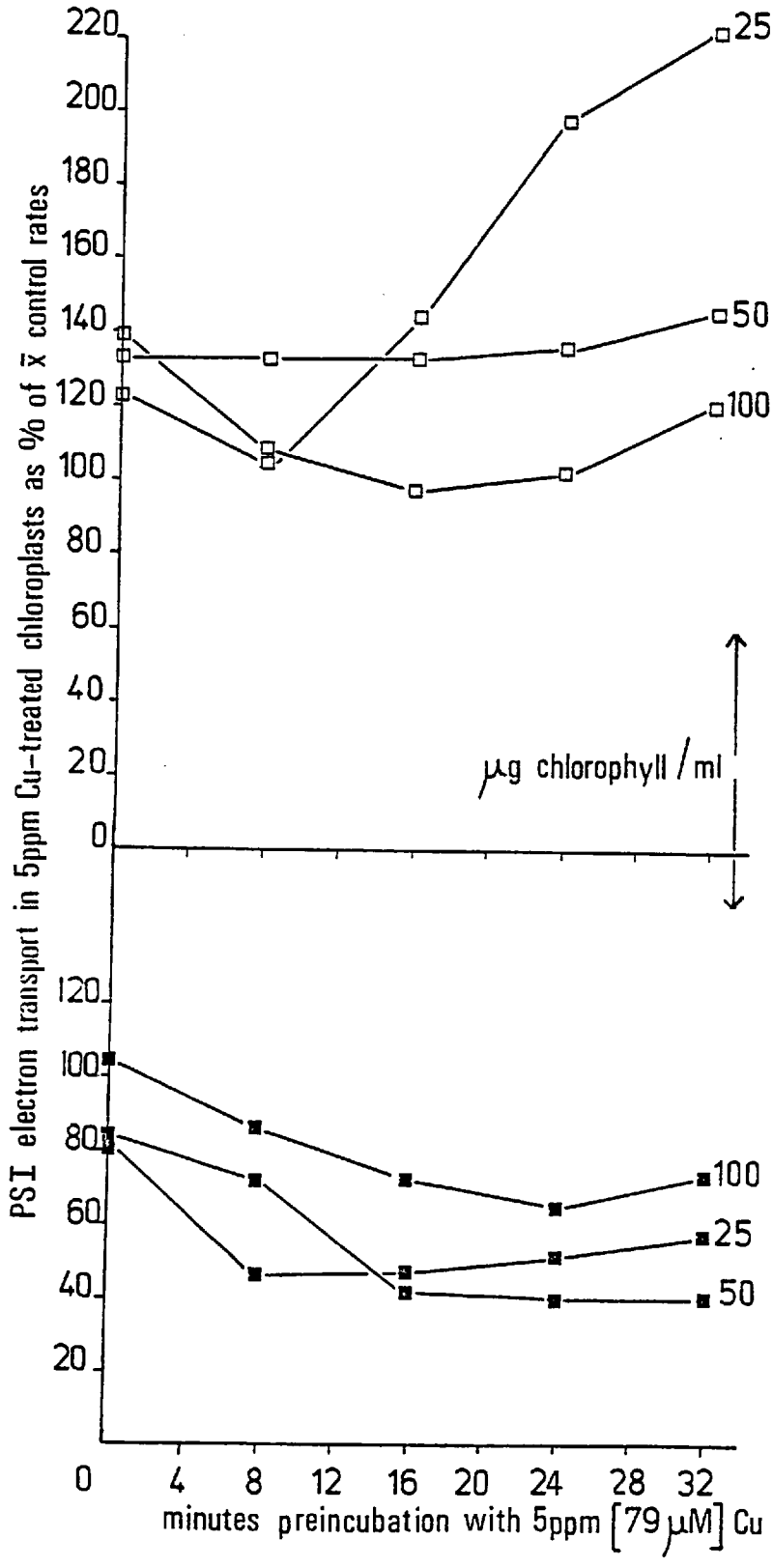


Figure 31. Effects of 5 ppm (79 µM) copper on coupled (□) and uncoupled (■) rates of PS I O_2 uptake over time in chloroplast suspensions containing various chlorophyll concentrations.

To try and further clarify this possibility of copper acting as an uncoupler , calculations were made to express the coupled and uncoupled rates as a ratio, the values initially being expressed as a percentage of the appropiate $\bar{x}$ control rate. The resultsare given in Table XII.

TABLE XII Ratios of 5 ppm Cu-treated PS I coupled to uncoupled rates at three different chlorophyll concentrations.
(Each individual value was initially expressed as a % of an appropiate mean control rate).

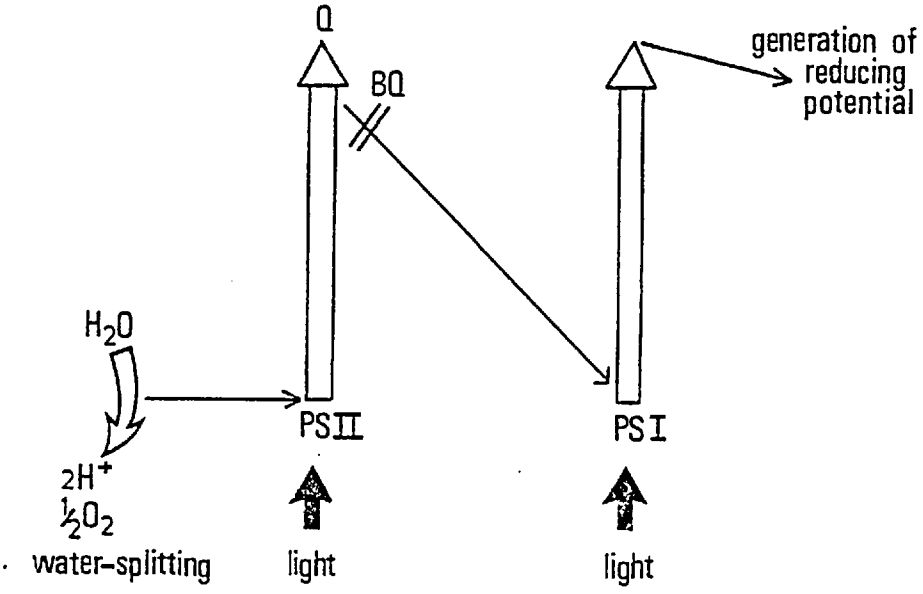
Mins. preincubation in 5 ppm Cu	Chlorophyll Conc. (µg/ml) 25	50	100
0	1.5	1.6	1.3
8	2.3	1.9	1.3
16	3.0	3.2	1.4
24	3.9	3.4	1.6
32	3.9	3.6	1.6

There appeared to be little difference between the sets of data for the 25 and 50 µg chlorophyll/ml treatments. This might indicate the operation of a threshold effect vis-a-vis a critical amount of metal required per thylakoid for the uncoupling effect to occur. The data for the 100 µg/ml treatment, however, is distinctly seperate. We are essentially comparing the coupled rate as a percentage of its $\bar{x}$ control rate with the uncoupled rate as a percentage of its $\bar{x}$ control; if neither of the two diverge far from controls (i.e. stay at around 100%), then the ratio will remain at approximately 1.0. The fact that, for 100 µg chlorophyll per ml, the coupled:uncoupled ratio only varied between 1.3 and 1.6, while for 25 µg/ml it went from 1.5 to 3.9, can therefore be taken as evidence that the copper is causing far less stimulation of coupled rates with more chloroplasts present than it is with a lower level.

(iii) Effects of copper on PS II electron transport.

In the system used for PS II assessment, the artifical electron acceptor used was benzoquinone (BQ). This compound picks up electrons at a point soon after substance Q which is reduced by the electrons flowing through PS II. Figure 32 shows the point of BQ involvement in the "Z" scheme.

Figure 32 Outline of the system employed for PS II assessment.



The acceptance of electrons by BQ enables the water-splitting event to release oxygen and one therefore monitors the biochemical behaviour of the system by reference to oxygen evolution. Coupled control rates of oxygen evolution were typically approximately 70 μM O_2/mg chlorophyll/hour, with a 3 to 4 fold increase after uncoupling with NH_4Cl.

Table XIII displays the coupled data from preincubation experiments in which a range of copper concentrations up to 30 ppm (476 μM) were employed and PS II rates assessed at intervals.

TABLE XIII Coupled rates of PS II O_2 evolution in Cu-treated chloroplasts preincubated in the light.
(Result are expressed as a percentage of controls).

Mins. preincubation in the light	Copper treatment (ppm)							
	1	2	4	5	6	7	10	30
0	88.7	64.9	89.2	90.8	87.8	80.5	70.6	50.0
5	91.6	78.6	70.6	86.2	71.8	60.9	51.4	66.3
10	91.3	82.7	73.2	86.3	64.7	50.5	47.5	44.3
15	94.6	82.1	75.9	76.6	63.2	47.8	39.7	53.5
20	93.9	79.8	74.5	80.3	60.3	45.9	39.0	39.3
Overall $\bar{x}$ Rate	92.0	77.6	76.7	84.0	69.6	57.1	49.6	50.7

These results show a trend towards greater inhibition with higher copper doses, although again the picture is somewhat confused by uncoupling. With 1 and 2 ppm treatments, there is an actual rise in rates between the 0 and 20 minute sampling times, but this trend becomes progressively less well marked as

the metal concentration rises. By looking at the $\bar{x}$ rates of O_2 uptake over the entire experimental period, one can gain an idea of the overall progressive decline.

Figure 33 depicts the associated uncoupled data. Here, the various traces followed a neat order with more copper causing increased inhibition. It was often the case that much of the effect of a particular treatment was already visible by the "time zero" sample, but there was also generally a decline in rates over the period of the experiment, indicating a progressive toxicological action.

Figure 34 plots the mean rate of oxygen evolution for both coupled and uncoupled chloroplasts, over the whole experimental period, as a function of the copper dose. The traces reinforce previous data which showed that uncoupled rates are more sensitive to copper inhibition than are coupled. When considering absolute rates of transport, it was noticed that, with moderate copper treatments of up to about 5 or 6 ppm, even after the twenty minute preincubation period, uncoupled rates were still significantly higher than coupled. Over the range of approximately 7 to 10 ppm copper, the final rates were roughly the same for coupled and uncoupled chloroplasts, while with still higher metal doses, the final uncoupled rates were generally lower than the coupled.

Figure 35 replots some of the above data alongside the results of an experiment in which the preincubation was carried out in the dark. The traces show that, in coupled chloroplasts, there was a trend for dark preincubation to reduce copper's inhibitory effects when compared to treatment in the light. However, at 10 ppm, the two traces virtually merged towards the end of the experimental period and, at 30 ppm, the light trace was least affected by the metal. Although the inhibition was again apparently more severe for any given metal treatment with uncoupled chloroplasts than with coupled, a virtually identical overall pattern emerged as that outlined above; there was less supression of rates in the dark, but the two 30 ppm traces were more or less identical with almost total inhibition.

A final experiment compared the effects of 5 ppm (79 μM) copper on PS II rates with three different chlorophyll concentrations present in the reaction medium. The results are given as Table XIV.

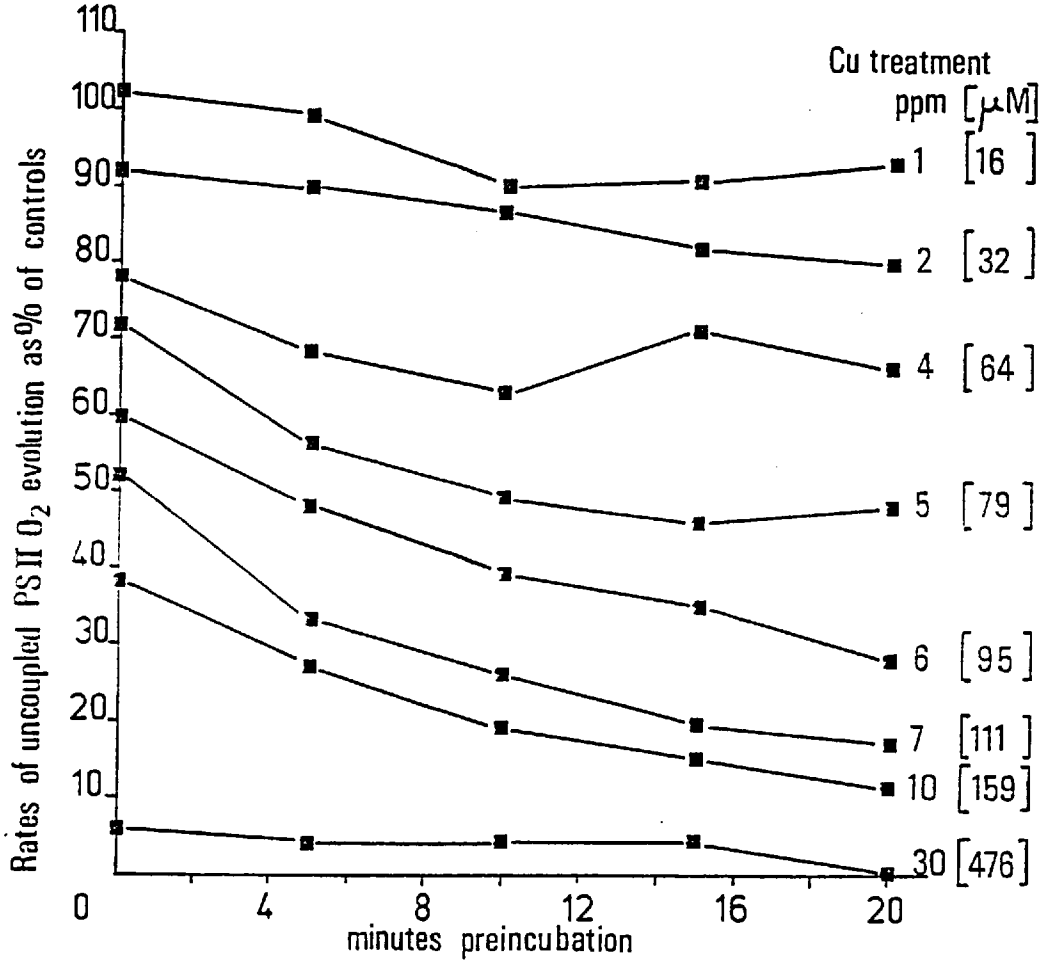


Figure 33. Effects of a range of copper concentrations on uncoupled rates of PS II O_2 evolution. Rates are expressed as a % of controls.

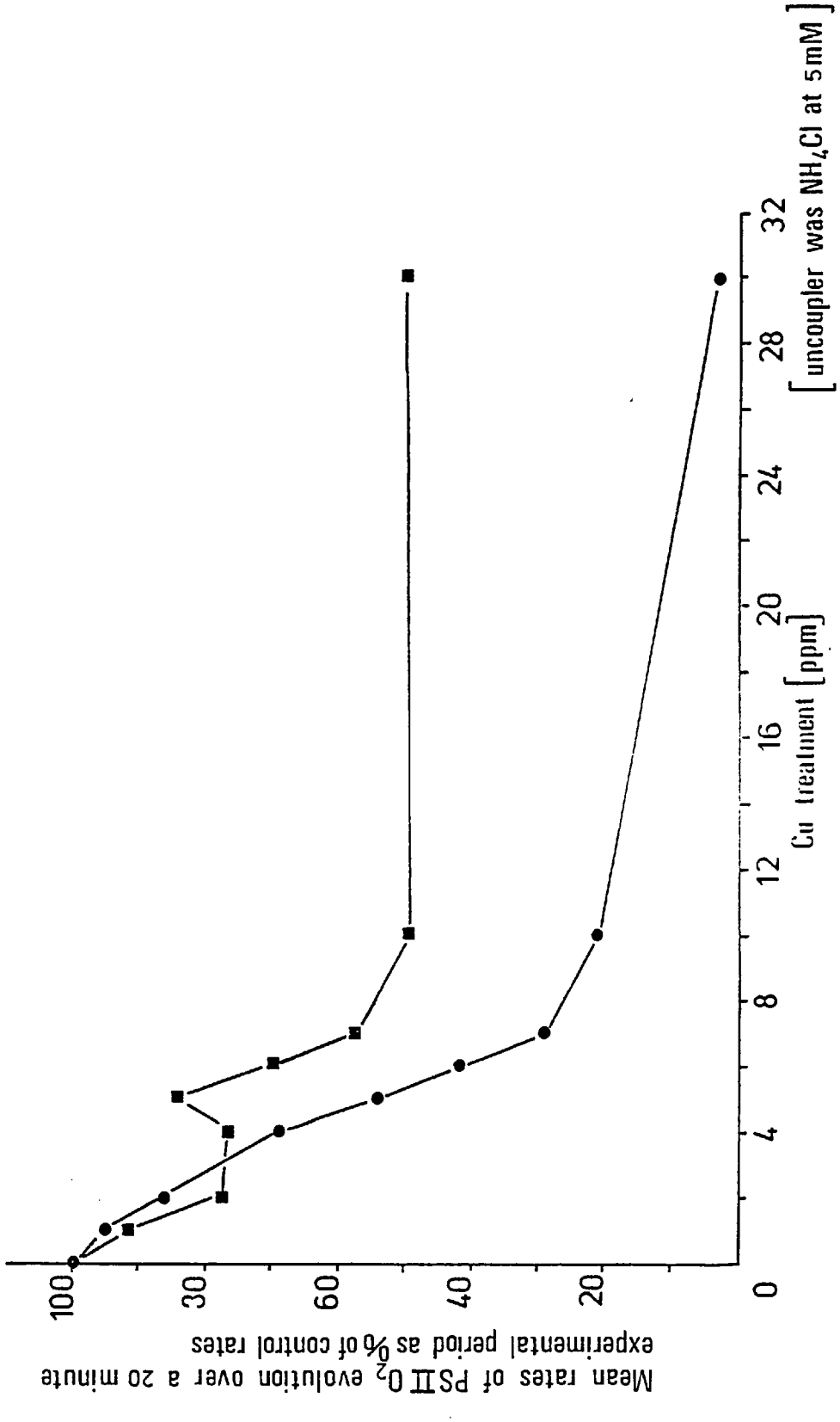


Figure 34. A comparison of the effects of a range of copper treatments on rates of PS II evolution in both coupled and uncoupled Chloroplasts. (■) (●)

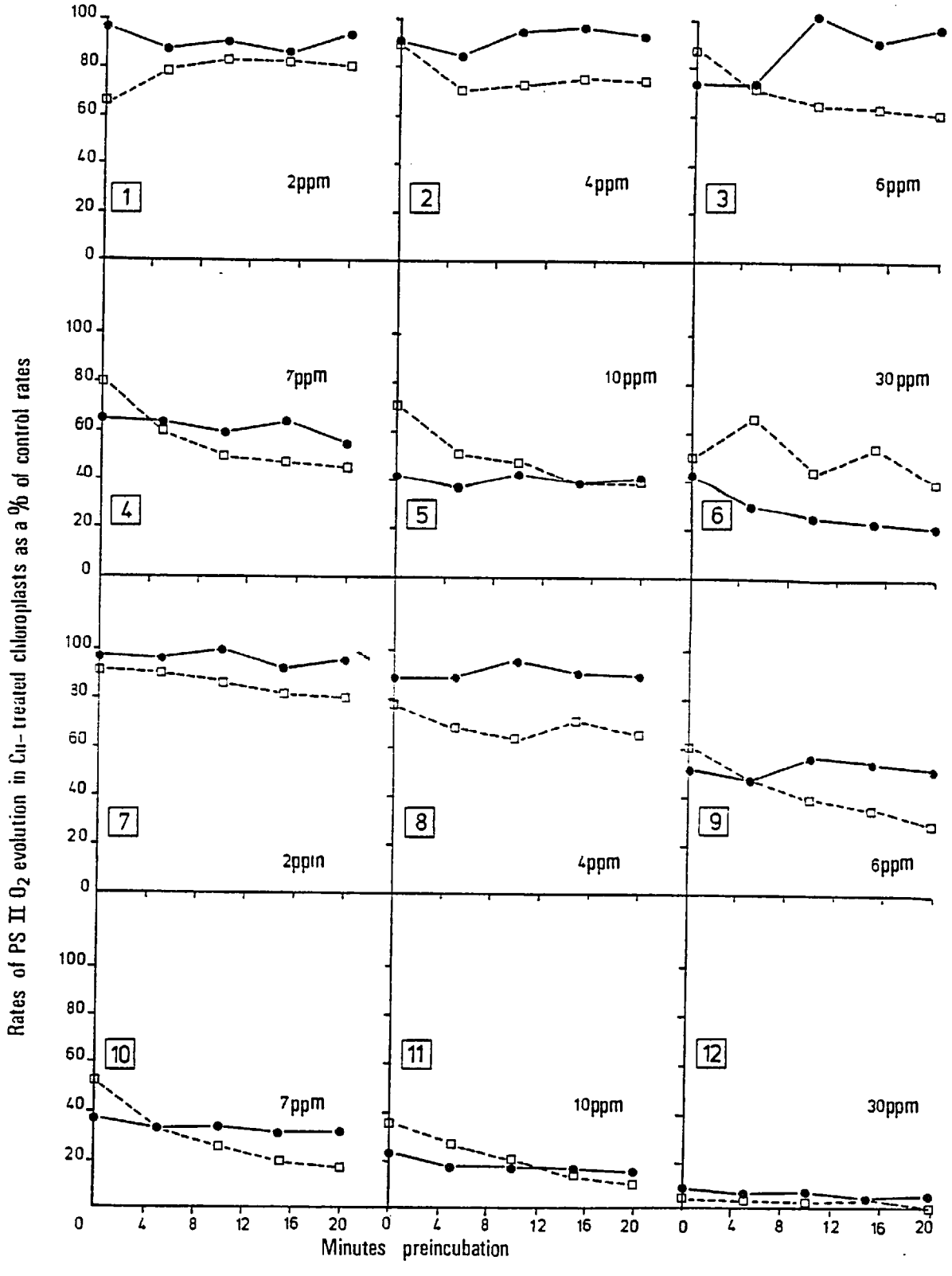


Figure 35. Effects of varicus copper treatments on coupled (graphs 1 to 6) and uncoupled (graphs 7 to 12) rates of PS II O_2 evolution in chloroplasts preincubated in the light (□) and the dark (■). Results are expressed as a % of control rates.

TABLE XIV Effects of 5 ppm (79 μM) on PS II O_2 evolution in reaction mixtures containing three seperate chlorophyll concentrations. (Results are expressed as a percentage of controls).

Minutes preincubation	Chlorophyll concentration (μg/ml) 25		50		100	
	Coupled	Uncoupled	Coupled	Uncoupled	Coupled	Uncoupled
0	106	44	90	53	93	64
5	100	37	83	49	75	66
10	101	32	77	45	73	61
15	100	24	76	36	73	55
25	86	20	74	32	72	55
Overall $\bar{x}$ rate	99	31	80	43	77	60

These results seem to clearly indicate that increasing the chlorophyll concentration results in increasing inhibition of coupled rates of PS II O_2 evolution. Conversely, higher levels of chlorophyll presence when the chloroplasts are uncoupled causes increasingly less inhibition. This can again be interpreted as further, more definite, evidence that copper is in some way causing uncoupling.

(iv) Comparison of the effects of copper on photosystems I and II.

Figure 36 combines some data, from experiments already reported above, so that a comparison can be made of copper's effects on photosystems I and II. Treatments of 1, 5 and 10 ppm are included, and the values all refer to uncoupled rates. The main point to note is that, in all three cases, the trace for PS II is well below that for PS I, indicating the former to be far more susceptible to inhibition by copper than the latter.

When the same type of comparison was made to look at the effects of three different chlorophyll concentrations (figure 37), the same overall picture emerged. In each of the treatments, photosystem II suffered most severe inhibition.

(v) Effects of copper on the 518 nm chlorophyll absorption shift.

The time taken for half of the 518 nm absorption peak of isolated broken chloroplasts to decay away was calculated. The thylakoids were preincubated for

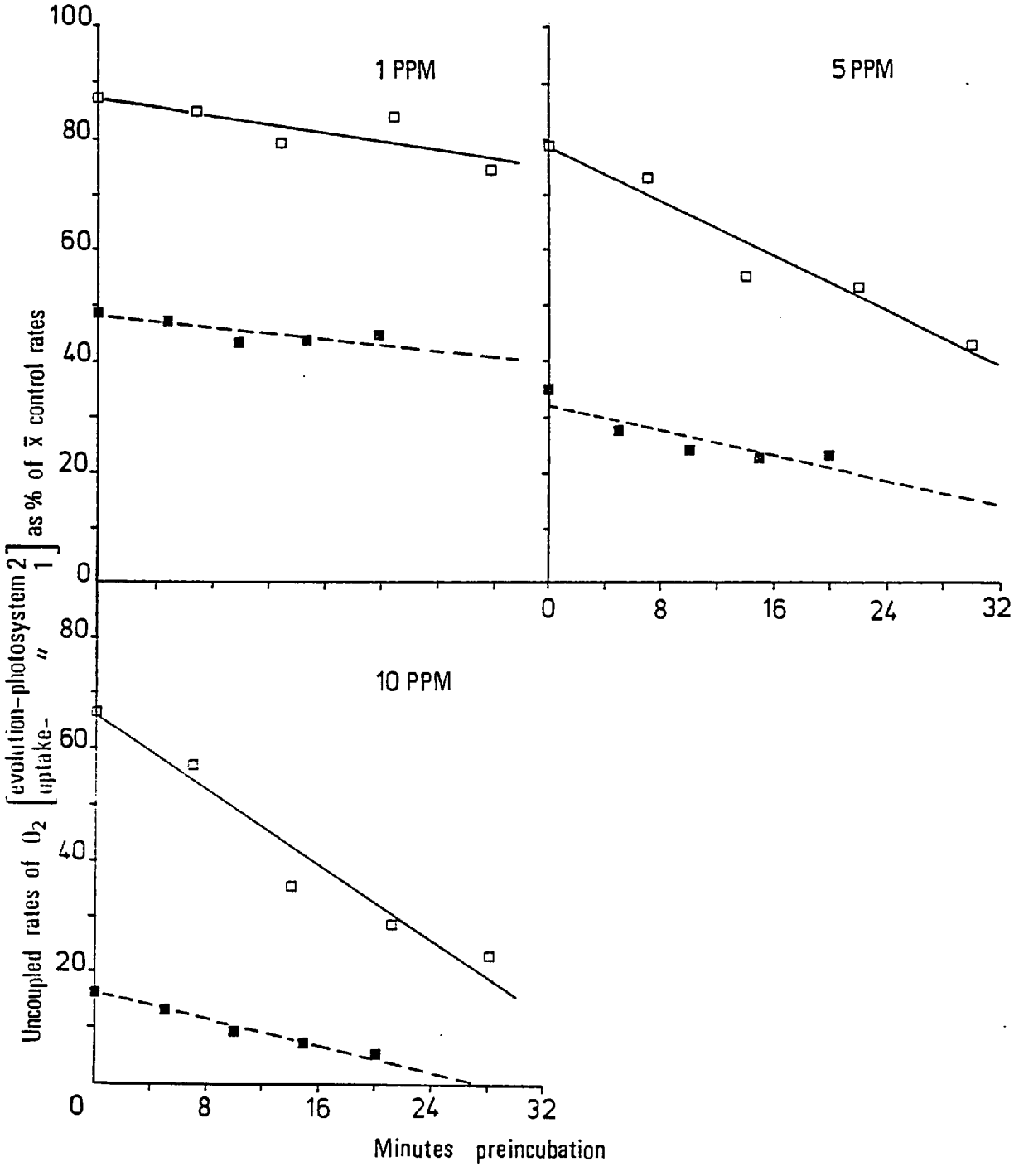


Figure 36. Effects of three different copper concentrations on uncoupled rates of electron transfer in PS I (□) and PS II (■) active chloroplasts. Results are expressed as a % of mean control rates.

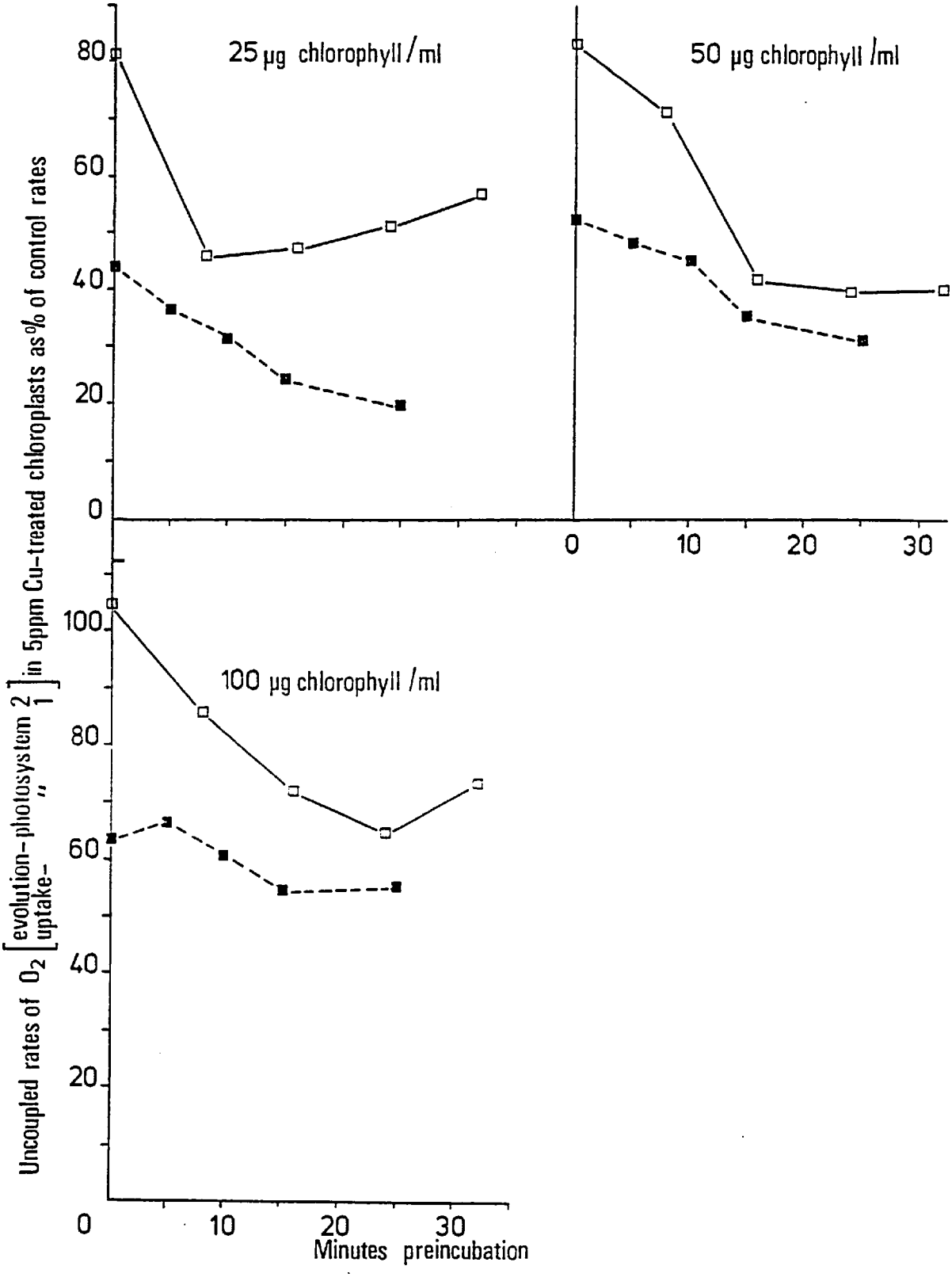


Figure 37. The effects over time of a 5 ppm (79 µM) copper treatment on PS I (□) and PS II (■) with three different chlorophyll concentrations present in the reaction medium. Results are expressed as a % of control rates.

times of up to 30 minutes, and results for control and copper-treated material were obtained. The data is presented as figure 38 and 39.

Figure 38 shows some of the actual absorption traces produced. The top two graphs are for initial control treatments after two different preincubation times. It was found that these control chloroplasts gave a $T_{\frac{1}{2}}$ value of 227 msec. at 0 minutes, and 115 msec. at plus 20 minutes. Thus, over this period, the time taken for half of the absorption peak to decay away was reduced by roughly 50%. The lower two traces are for 3.33 ppm copper treatments after 0.5 and 20 minute preincubation periods. By the 0.5 minute point, the $T_{\frac{1}{2}}$ value had already declined to 124 msec., and at plus 20 minutes, it was down to 44 msec. A much increased degree of uncoupling of the chloroplasts was therefore indicated in the case of the copper-treated material.

Figure 39 plots the various $T_{\frac{1}{2}}$ values obtained for chloroplasts treated with copper levels of up to 20 ppm against the time of preincubation. It can be seen that the trace for the final control lies below that for the initial control; this reflects the natural ageing processes of the chloroplasts. They had been isolated 3 to 4 hours previously and their degree of uncoupling had gradually increased. However, the main point of note is that the traces for 3.33, 10 and 20 ppm-treated chloroplasts, which were produced before that for the final controls, are all below both of the control lines. This is clear evidence that, in all three cases, the copper has increased the degree of uncoupling of the chloroplasts by a substantial amount, and thereby given rise to the much increased rate of decay of the 518 nm absorption peak. The depressed trace for the final control indicates that, to allow for natural ageing, the lines for copper-treated chloroplasts should be revised upwards slightly. However, they would still fall significantly below the control trace, indicating increased uncoupling.

The three copper concentrations employed seem to have had broadly similar effects on the rate of decay of the absorption peaks. This indicates that perhaps some sort of threshold effect was operating and that 3.33 ppm was already a sufficiently high enough level to cause significant uncoupling.

3.3b Photosystem II chlorophyll fluorescence work.

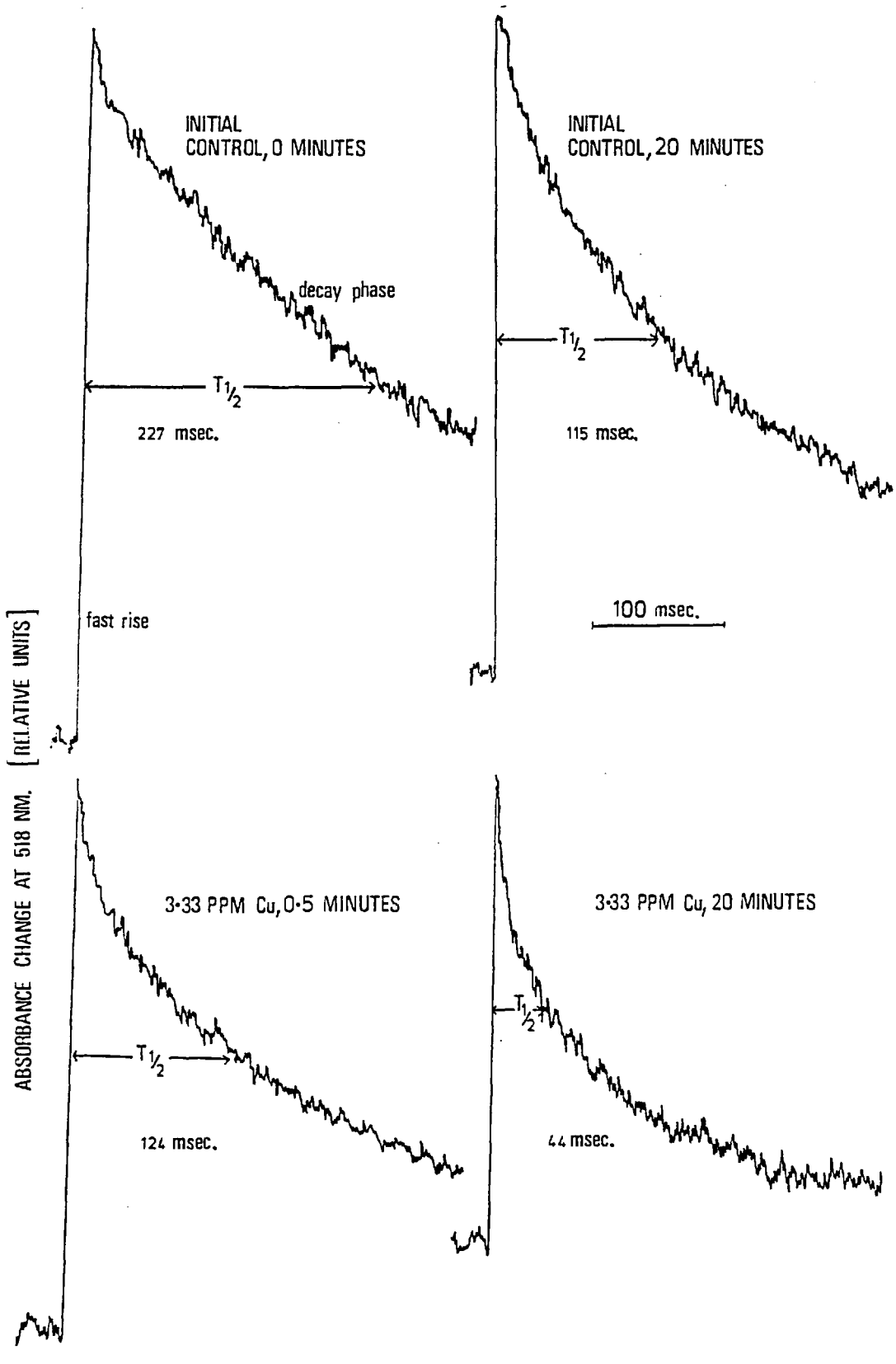


Figure 38. Decay of the 518 nm absorption peak. The $T_{\frac{1}{2}}$ values for the decay phase are given for control chloroplasts (top two traces) after 0 and 20 minute preincubation times and for 3.33 ppm copper-damaged material (bottom two traces) after 0.5 and 20 minutes of treatment.

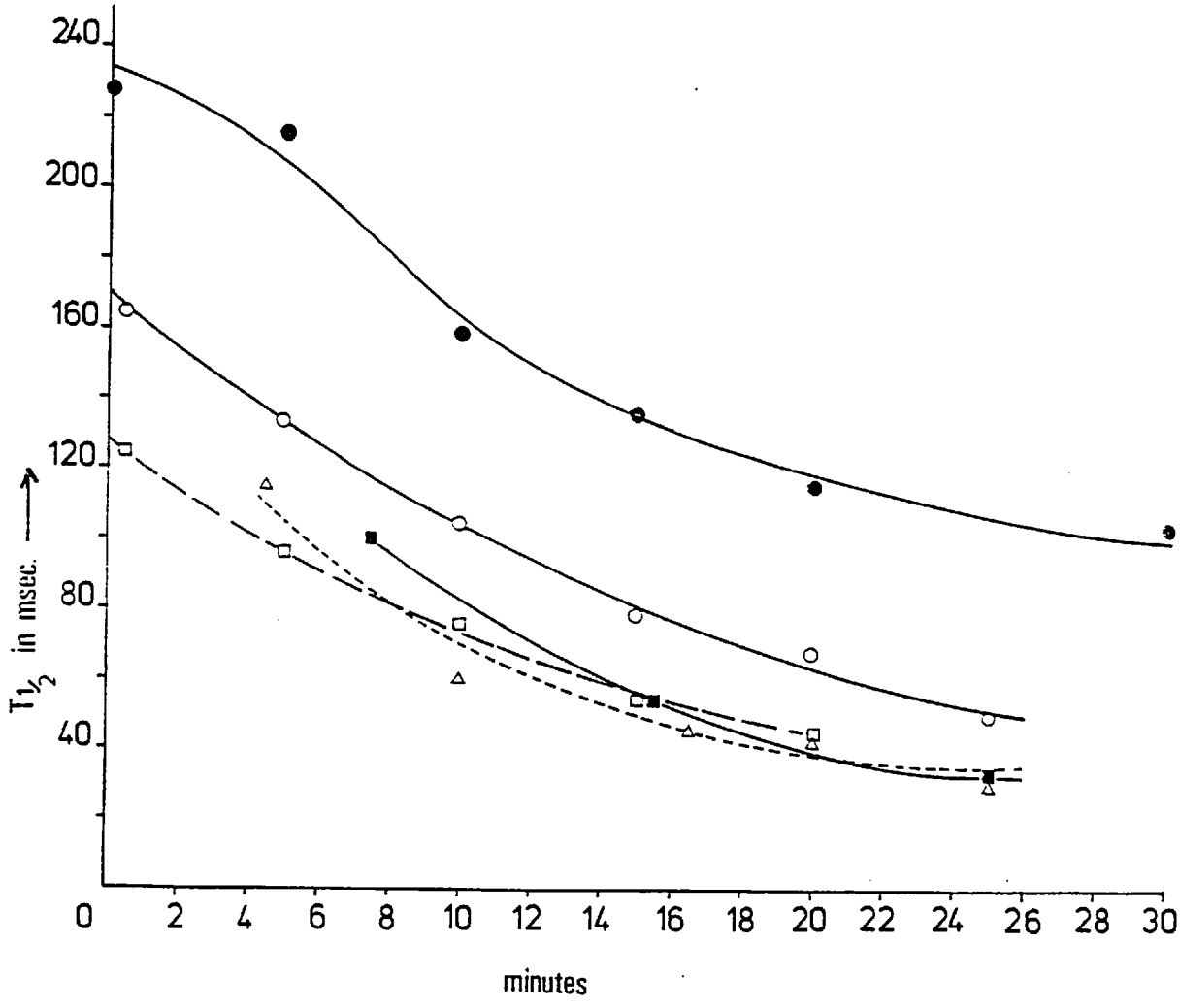


Figure 39. Decay of the 518 nm absorption peak over time in isolated chloroplasts subjected to various copper treatments. The $T_{\frac{1}{2}}$ value in msec. is a measure of the decay phase. (●, initial control: ○, final control: ■, 10 ppm Cu: □, 3.33 ppm Cu and △, 20 ppm Cu).

(i) Experiments with isolated chloroplasts.

The level of chlorophyll fluorescence yield from photosynthetic tissue at room temperature depends on the degree of reduction of the primary electron acceptor of photosystem II, termed Q by Duysens and Sweers, 1963 and Duysens, 1963. By monitoring the level of fluorescence, one can therefore obtain an indirect measure of the magnitude of the electron flow to Q, relative to controls. By elucidating the characteristics of the fluorescence inhibition obtained after, say, a copper treatment, and by then trying to restore the levels to normal with the use of artificial electron donors, one can try to develop a picture of the site of copper's inhibitory action.

The first experiment to be conducted looked at copper's effects on steady-state fluorescence levels. In each case, a control stready-state fluorescence yield was obtained and then the metal was added; the final yield was noted when the trace became as steady as possible (DCMU was not present). Figure 40 contains a selection of the graph traces produced. A clear progression was demonstrated whereby the fluorescence yield ultimately obtained was progressively lower with increasing metal treatments. Table XV summarises the data from these traces and also includes parallel figures obtained using cadmium treatments. This can provide a comparison of the severity of the effects on fluorescence of the two heavy metals.

TABLE XV Inhibition of final steady-state fluorescence in Cu and Cd-treated chloroplasts.
(Results are in each case expressed as a percentage of the initial control steady-state levels).

ppm metal	mM Cu	mM Cd	% inhibition of Cu-treated chloroplasts	% inhibition of Cd-treated chloroplasts
1.7	.03	.02	13	0
3.3	.05	.03	13	2
10.0	.16	.09	17	3
13.3	.21	.12	20	1
16.7	.27	.15	20	6
20.0	.32	.18	22	5
23.3	.37	.21	28	5
26.7	.43	.24	27	4
33.3	.53	.30	28	7
50.0	.80	.45	40	9
66.7	1.07	.59	45	16
100.0	1.60	.89	54	17
133.3	2.13	1.19	55	21
166.7	2.67	1.48	75	32
200.0	3.20	1.78	75	42

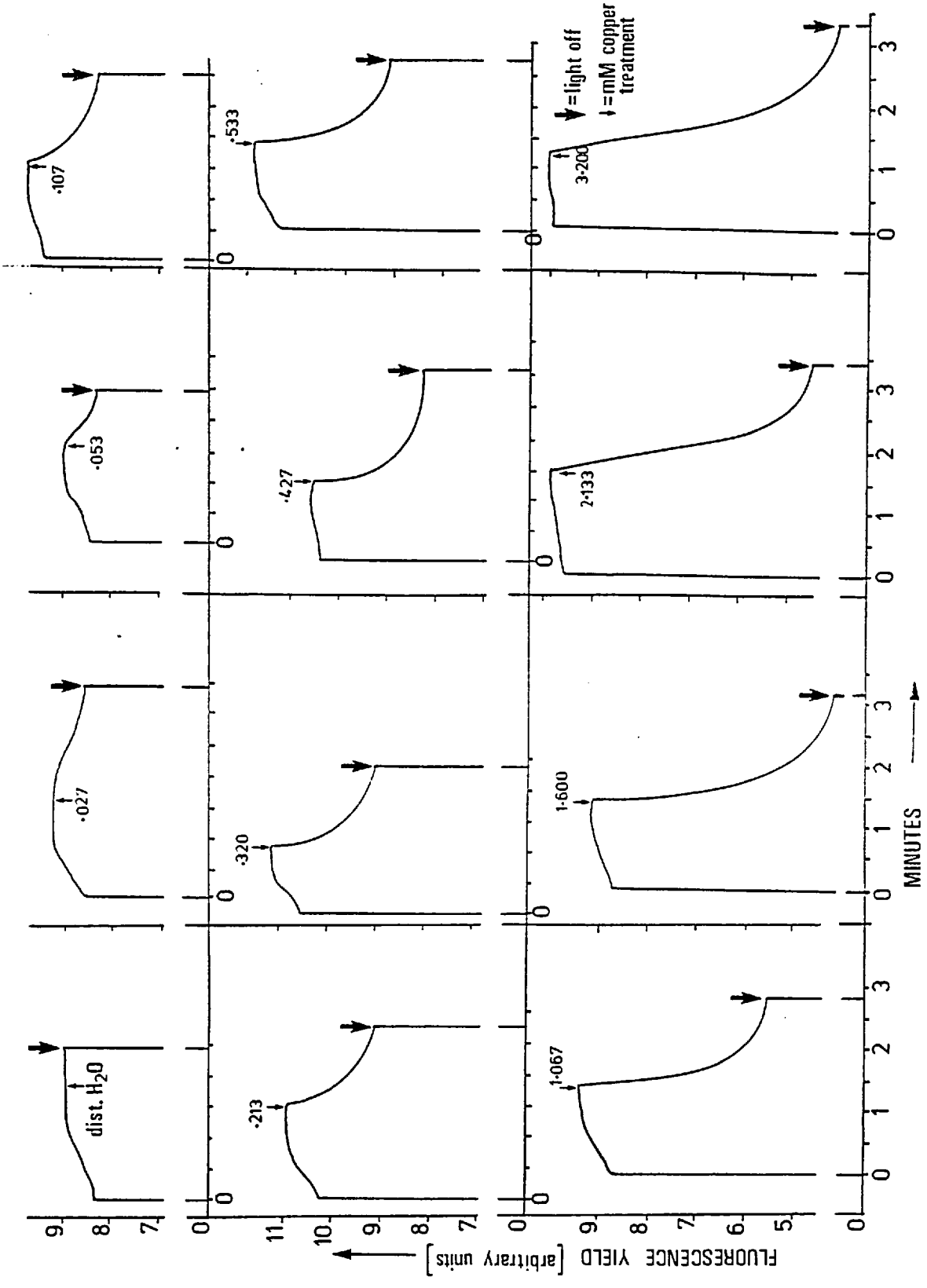


Figure 40. The effects of a range of copper concentrations on PS II fluorescence of isolated chloroplasts. No DCMU was present.

The results indicate that, on a ppm basis, PS II fluorescence, is far more sensitive to inhibition by copper than it is to cadmium. Only 1 or 2% inhibition was caused by cadmium levels of up to 13.3 ppm, while a similar copper concentration had caused a 20% reduction in yield. At higher metal levels (100 ppm and upwards), the difference in the inhibitions caused by the two metals was reduced, but still equal to variation by a factor of at least 2 or 3. Even on a mM basis, a comparison of the effects of the two metals indicates a lesser toxicity caused by cadmium than by copper.

An experiment was conducted to investigate whether the presence of cations (supplied as $MgCl_2$) in the reaction medium would have any effect on the ability of the metals (Cu and Cd) to inhibit fluorescence. The cations would put the chloroplasts into the high fluorescence state. This may be because their presence reduces the so-called spill-over of excitons from PS II to PS I, thereby helping to keep Q in a reduced form. HOMANN (1969) also hypothesized that Mg^{2+} ions facilitate an activation of Qi, an inactive form, to Qa which permits a more complete photoreduction of the overall Q pool. MURATA (1969 and 1970) said that these cation-induced fluorescence changes were brought about by changes in the membrane structure of the chloroplast lamellae. BARBER has reviewed more recent studies and observations relevant to this effect (C.I.B.A. foundation, 1979 and BARBER, 1976). Table XVI summarises the results obtained in these studies.

TABLE XVI Initial fluorescence yields in Cu and Cd-treated chloroplasts, with and without cations present.
(Results are expressed as a percentage of controls).

Metal concentration (mM)	Copper		Cadmium	
	With cations	No cations	With cations	No cations
0	100	100	100	100
.03	-	-	100	96
.05	90	83	-	-
.15	-	-	105	95
.27	82	74	-	-
.29	-	-	104	111
.52	75	54	-	-
.60	-	-	97	103
.89	-	-	95	100
1.06	63	48	-	-
1.18	-	-	92	93
1.49	-	-	83	90
1.59	48	38	-	-
1.80	-	-	90	95
2.11	45	35	-	-
2.20	-	-	84	92
2.65	41	37	-	-

These results seemed to demonstrate that, for copper, there was consistently slightly less inhibition when cations were present than when they were not. If anything, the trend was found to be the opposite when cadmium was used, although here the differences were less marked. Even with copper, the differences between the two sets of figures became progressively less well pronounced as the metal concentration increased. DCMU was present throughout.

A similar experiment was conducted to look at the relevance of DCMU presence on copper's inhibitory effects. Mg Cl_2 was present throughout and the results produced are given below as Table XVII.

TABLE XVII Initial fluorescence yields in Cu-treated chloroplasts with and without DCMU present.
(Results are expressed as a % of control yields).

Cu concentration (mM)	With DCMU	No DCMU
0	100	100
.05	84	83
.27	76	72
.53	67	66
1.06	51	51
1.59	47	43
2.12	42	43
2.65	38	40

No real differences in response to the two treatments could be distinguished and therefore the presence of DCMU seemed irrelevant to the inhibition of fluorescence by copper.

A comparison was made of the responses of normal and tris-washed chloroplasts to the addition of various artificial electron donation systems. YAMASHITA and BUTLER (1968) had shown that tris inhibited the Hill reaction in chloroplasts by blocking electron transfer between water and PS II, and HOMANN (1968) said that such washing, or a heat treatment, released 65 to 85% of the bound manganese from the chloroplasts. In 1969, YAMASHITA and BUTLER took the work further by showing that, after the tris inhibition, fluorescence yields could be restored by the addition of various artificial electron donors.

It was hoped that, if the tris-treated chloroplasts still exhibited copper-

sensitive fluorescence changes, then the subsequent use of artificial electron donors would help to elucidate the points at which the metal was inhibiting electron flow. Table XVIII and figure 41 summarise the fluorescence responses of normal and tris-treated chloroplasts to the addition of various of these donors.

TABLE XVIII Fluorescence levels in normal and tris-washed chloroplasts after the addition of various artificial electron donation systems, and then DCMU.
(All results are expressed as a % of the initial levels with just Mg Cl_2 present. In each case, the first figure is the fluorescence level after donor addition, and the second after then adding DCMU).

Donor system employed	Normal Chloroplasts	Tris-treated Chloroplasts
DCMU	100	174
Hydroxylamine, DCMU	99, 99	126, 179
$MnCl_2$, DCMU	101, 99	104, 169
Diphenylcarbazide, DCMU	108, 103	145, 165
Phenylenediamine plus ascorbate, DCMU	96, 91	148, 201
Acetone, DCMU	100, 100	111, 131
Distilled water, DCMU	100, 98	100, 151
Ascorbate, DCMU	93, 89	101, 170

The results showed that it was only with tris-washed chloroplasts that any real response to the artificial donors used was obtained. DCMU itself caused a large rise in fluorescence yield in the tris-washed chloroplasts (but no effect on normal). Figure 42 shows some of the actual traces obtained during these experiments.

The results indicate that, in tris-treated chloroplasts, there is only a slow donation of electrons to substance Q. This means that the Q pool cannot be fully reduced, and therefore fluorescence yields cannot rise to the maximum, until DCMU is added to block the flow of electrons out of Q. The addition of the various donors used also seemed to be able to partially boost the reduced fluorescence levels by causing extra electrons to flow through the system.

The effectivity of copper as a fluorescence inhibitor was then compared in normal and tris-washed chloroplasts. Table XIX gives the results. An initial level of fluorescence was gained for each sample and then copper was added; the fluorescence level 2 minutes after the copper addition was then expressed as a

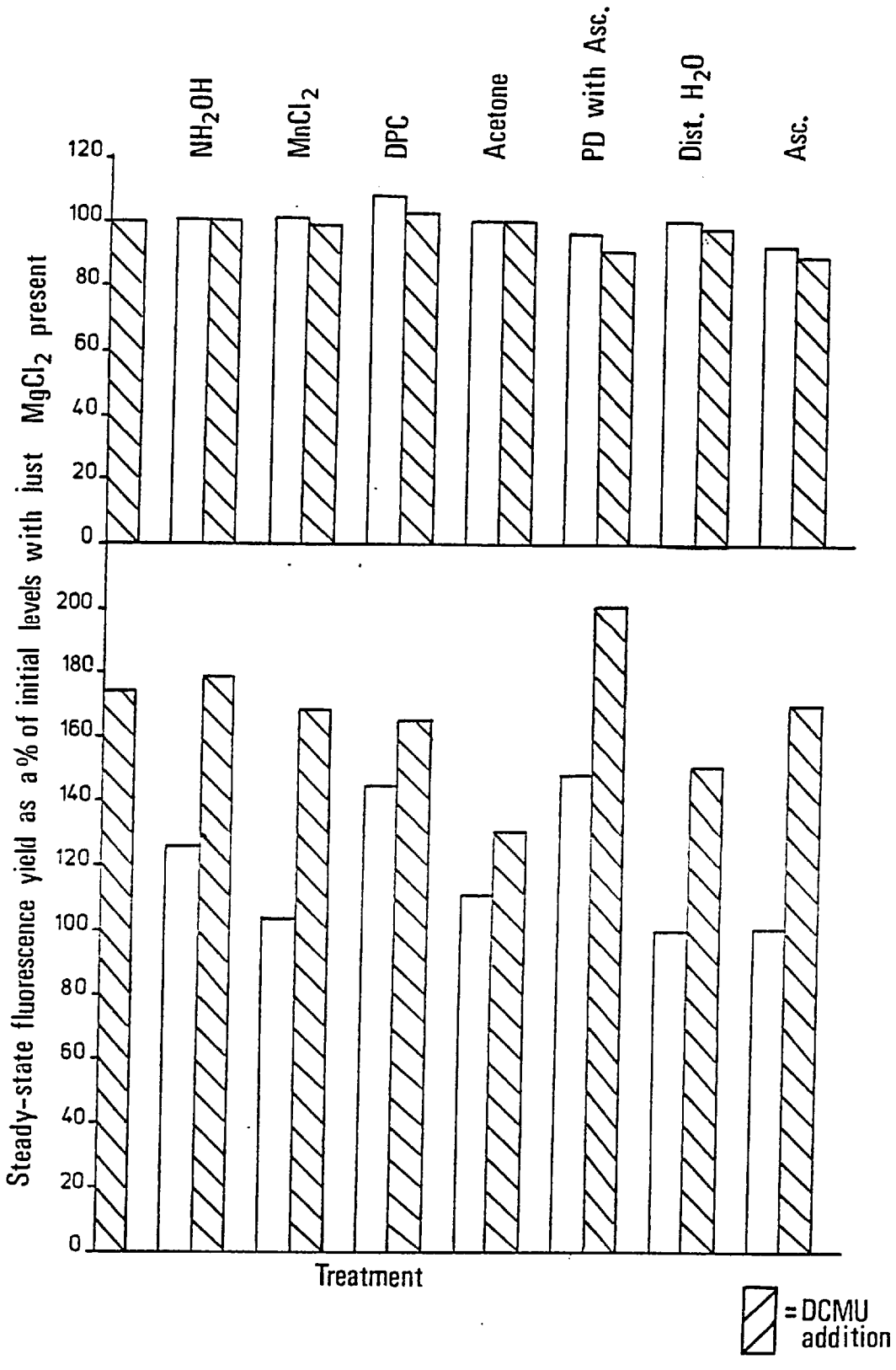


Figure 41. Fluorescence levels in normal (top histogram) and tris-washed chloroplasts (bottom histogram) after the addition of various artificial electron donors, and then DCMU. Results are expressed as a % of initial control levels with just $MgCl_2$ present.

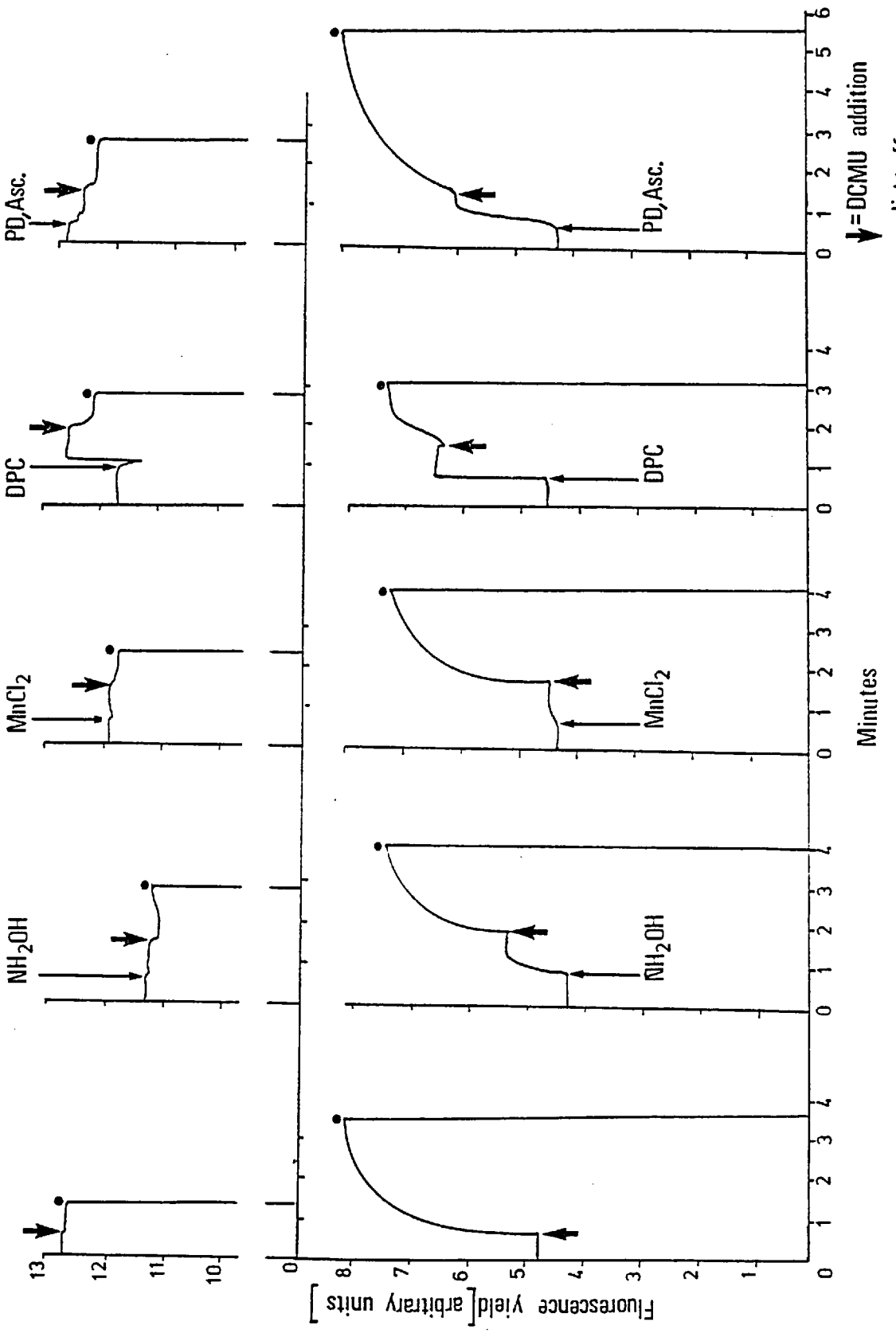


Figure 42. The effects of various artificial electron donor systems, followed by DCMU, on the fluorescence yield of normal (left hand) and tris-washed chloroplasts (right hand).

percentage of the initial yield.

TABLE XIX Fluorescence levels in Cu-treated chloroplasts two minutes after the introduction of metal, results being expressed as a percentage of initial control levels.

Copper concentration (ppm)	(mM)	Normal Chloroplasts	Tris-treated Chloroplasts
0	0	100	100
0.33	.005	92	100
1.33	.021	69	100
6.67	.107	72	93
16.67	.267	62	78
33.33	.533	62	64
50.00	.800	41	69
66.67	1.067	39	53

It was therefore apparent that tris-washed chloroplasts are progressively inhibited by increasing copper levels, but that they are less sensitive than are normal chloroplasts.

Figure 43 shows the traces obtained in a seperate experiment with tris-washed chloroplasts. Here, a steady control level of fluorescence was allowed to develop and then the copper was added. Two minutes later, DCMU was injected to see if its presence would have any effect. The traces again demonstrated a progressively greater inhibition with increasing copper concentration. In addition, it was apparent that the DCMU-induced fluorescence rise was also lost as a result of the copper treatments, this being more noticeable with the higher metal levels. Copper was apparently blocking the slow electron flow to Q that had been demonstrated in Table XVIII.

An attampt was then made to test the effectiveness of the artificial electron donor system phenylenediamine plus ascorbate as a restorer of fluorescence in tris-washed chloroplasts following copper treatments. Various copper doses were added to chloroplasts with a steady control level of fluorescence and, two minutes later, PD + Asc. were added. The effects on fluorescence levels were noted and then DCMU was injected. Table XX contains the data from this experiment.

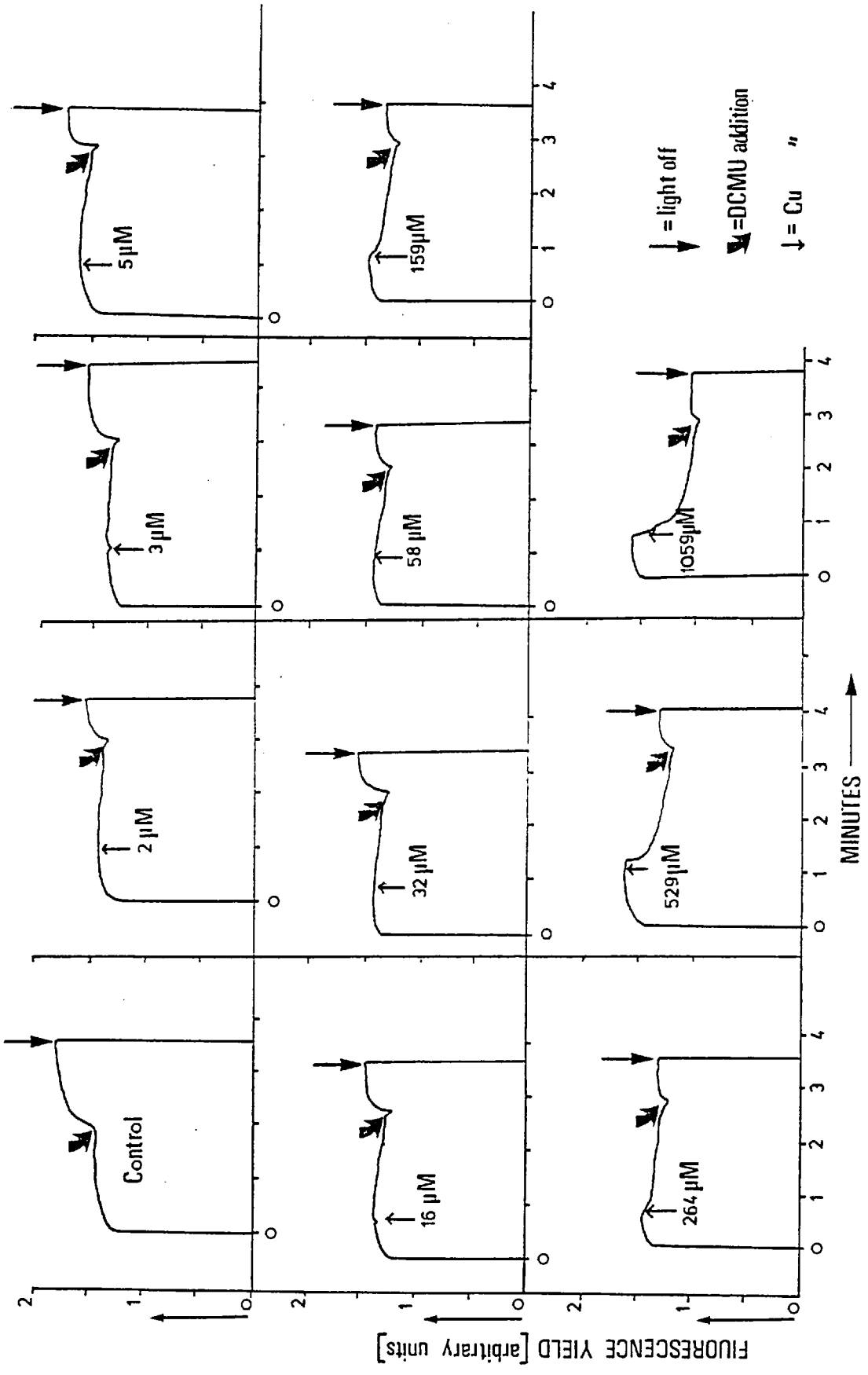


Figure 43. The effects of a range of copper concentrations, followed by DCMU addition, on the fluorescence yields of tris-washed chloroplasts.

TABLE XX Effectiveness of the donor system (phenylenediamine with ascorbate) as a restorer of fluorescence in Cu-treated tris-washed chloroplasts. Fluorescence yield as a percentage of the initial control level.

Copper treatment (mM)	Two minutes after copper treatment	After the addition of PD and Asc.	After the addition of DCMU
0	100	120	155
.003	98	97	114
.005	96	98	114
.010	96	104	110
.016	94	100	105
.058	92	92	92
.264	84	Addition of donor and DCMU only resulted in a continuing fluorescence decline as a result of the copper treatment.	
.529	78		
.794	78		
1.058	70		

The results indicated that only slight fluorescence restoration could be obtained using PD + Asc., and even then there was only a stimulatory effect at the lower copper levels employed. Thus, beyond 0.016 mM Cu (1 ppm), no restoration of the Cu-inhibited yield could be effected. Up to 0.016 mM Cu, the injection of DCMU had also further increased fluorescence yields, but this effect too was lost at higher metal concentrations. At 0.264 mM copper (16.5 ppm) and higher concentrations, the addition of PD and Asc., and also DCMU, only resulted in further decreases in fluorescence yield.

Experiments aiming to use hydroxylamine as a restorer of fluorescence employed a range of copper concentrations from 0.017 ppm (0.003 mM) to 66·67 ppm (1.058 mM). Cadmium levels from 3.33 ppm (0.030 mM) up to 33.33 ppm (0.300 mM) were also used. The metals were allowed to take effect for two minutes and then hydroxylamine (HA) was injected. Trials with both 5 mM and 50 mM HA proved totally ineffective in restoring fluorescence.

A similar result was obtained when attempts were made to use $MnCl_2$ as a donor. In order to maximise the chances of the donor being effective, only a low level (1.67 ppm, 0.027 mM) of copper was used, although a range of cadmium concentrations from 1.67 to 166.67 ppm was tried. $MnCl_2$ applications of from 85 µM to 10 mM were injected. At no time was $MnCl_2$ observed to cause any recovery of fluorescence levels after a copper treatment.

The use of diphenylcarbazide (DPC) in this type of work was precluded because of the formation of a violet compound whenever this electron donor was allowed to react with copper. As earlier work has shown, DPC could stimulate fluorescence in the control situation, in both normal and tris-washed chloroplasts, although more so in the latter, but the formation of the violet complex automatically resulted in an instantaneous apparent fluorescence drop to zero, and so its use with copper had to be abandoned. (N.B. Copper forms a violet coloured dithizonate when it reacts with diphenylcarbazone (dithizone) (KOLTHOFF and SANDELL, 1961) and RAMASWAMY and MADHUSUDANAN NAIR, 1978, reported a distinctive intense blue copper complex with bovine serum albumin).

(ii) Experiments with Chlorella cells.

Experiments using the fluorimeter.

A series of experiments was conducted to look at the fluorescence induction curves of Chlorella suspensions subjected to various copper treatments. The shape of the curves over the first few seconds of illumination was always much the same, the main point of note being that the initial peak yield was always reduced by a metal treatment. The experimental set-up did not have the time resolution to accurately investigate the very early kinetics of the fluorescence rise as indicated in figure 44. The 10 mM copper treatment employed was very high when compared to the concentrations relevant for the chloroplast work. This is because, when dealing with an intact organism, albeit a relatively simple one, relatively little of the applied inhibitor may reach sensitive sites. Using higher copper treatments (e.g. 20 mM), a progression of results could be obtained, whereby there was a graded response in the induction curves with time. Thus, a set of curves could be produced in which, as the time of preincubation increased, the yields progressively declined. However, although fluorescence levels fell, the shape of the traces remained more or less constant.

Experiments were conducted to investigate the effects of copper on the DCMU-induced fluorescence increase visible with Chlorella cells. The addition of DCMU to control cells caused an increase in the fluorescence yield, indicating that there is normally only a relatively slow rate of electron flow to the Q pool. Copper treatments were used to see if this part of the electron pathway was

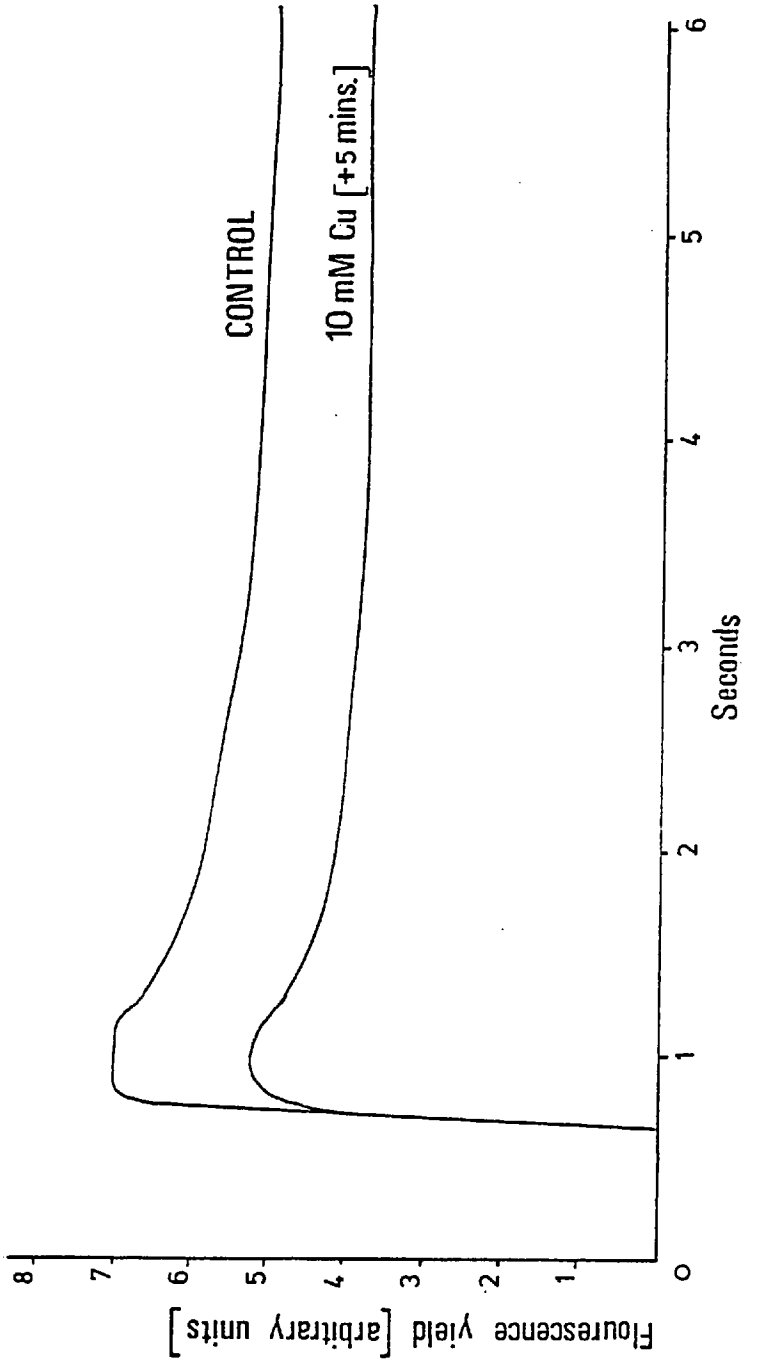


Figure 44. The first few seconds of the fluorescence response, on illumination, in control and copper-treated chloroplasts.

sensitive to metal inhibition. Cells were allowed to reach a control fluorescence level and then a 1 minute treatment with a particular copper concentration was administered. When the 1 minute had elapsed, DCMU was added and the resulting fluorescence yield expressed as a percentage of the initial control yield. The results obtained are displayed as figure 45. Over the range of 0 to 8 mM copper, a good straight line relationship was demonstrated with higher copper levels increasingly inhibiting the fluorescence stimulation obtainable on the addition of DCMU. Figure 46 includes some of the fluorescence induction surves (longer time scale than figure 44) obtained after the above copper treatments, on the addition of DCMU. Not only was the ultimate yield lower with increasing copper levels, but it also took longer for the steady levels to develop. (N.B. It may be here worth noting that, in many of the experiments conducted, a "steady" fluorescence level, obtained after a metal treatment, may really only be the yield reached at a point beyond which the decline is no longer noticeable in the short term. Particularly with the higher metal treatments, it must be true that, given time, the yield would probably eventually decay to a low level. This is because the copper would gradually affect all types of biochemical processes within the cell, thereby causing a general lessening of the rate of many different metabolic activities that would gradually have secondary, indirect effects on fluorescence).

That the dose level and time of exposure to metal are both of importance in fluorescence inhibition became obvious from several of the experiments already reported. A further experiment (see figure 47) underlined this fact. This figure gives some of the fluorescence traces obtained when DCMU was added to Chlorella samples that had been treated with 6 mM copper for a range of times up to 18 minutes. It can be seen that the ability of DCMU to enhance the fluorescence yield above initial control levels declined as the time of exposure to the metal increased. The time taken for the steady state level of fluorescence yield to be obtained also increased as the time of treatment became longer.

Further work was aimed to investigate the effectivity of the artificial electron donor hydroxylamine as a possible restorant of fluorescence levels following a metal treatment. Control fluorescence was followed by a 1 minute treatment with either 0, 2 or 6 mM copper. Then, either DCMU alone or DCMU plus HA were added and the effects on fluorescence yield noted. Table XXI details the results that emerged and figure 48 shows the traces for one replicate of each of 0 and 6 mM copper.

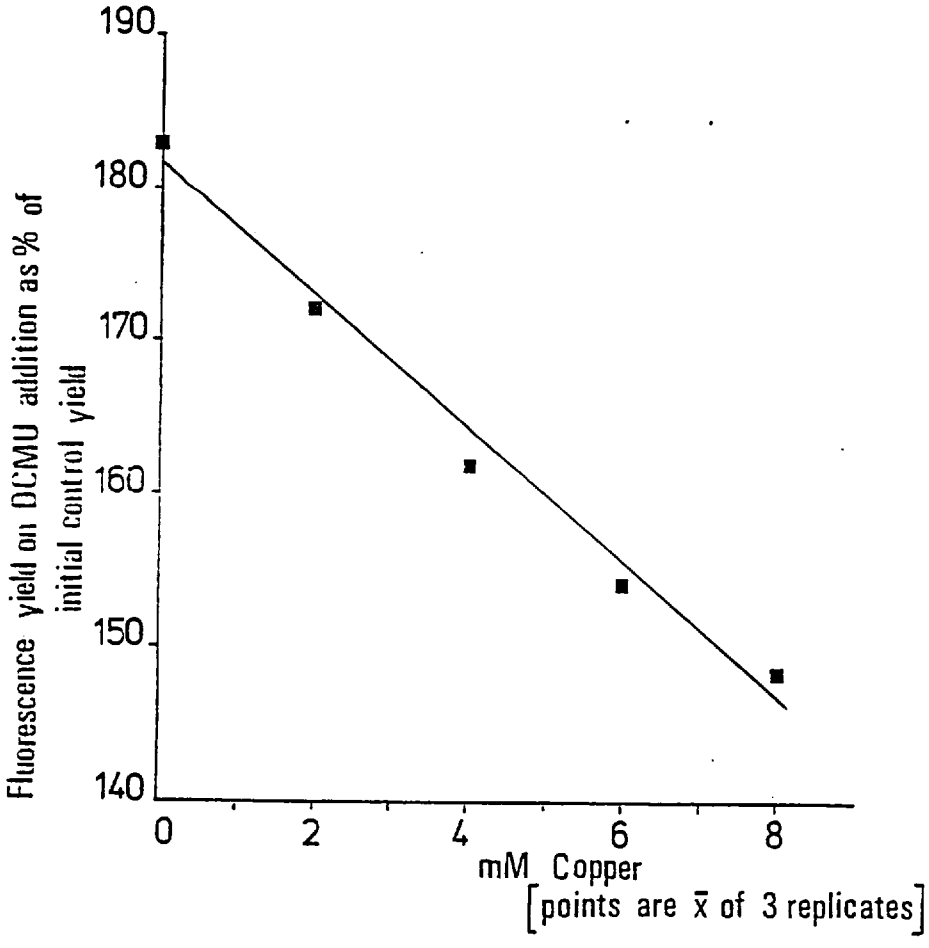


Figure 45. Fluorescence yields of isolated chloroplasts on DCMU addition after various copper treatments. Results are expressed as a % of initial control yields.

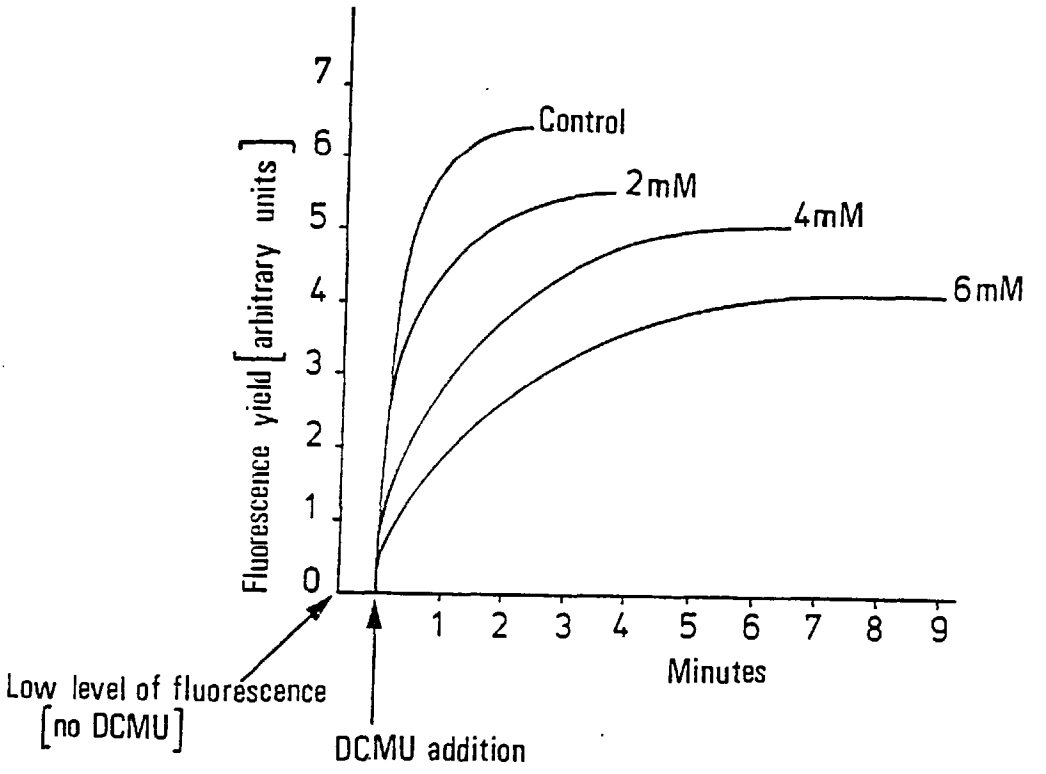


Figure 46. Fluorescence yields of Chlorella cells. (Two minutes were allowed for a steady control yield to develop and then a copper treatment was administered. One minute later, 10 μM DCMU was introduced and time allowed for the fluorescence yield to become steady).

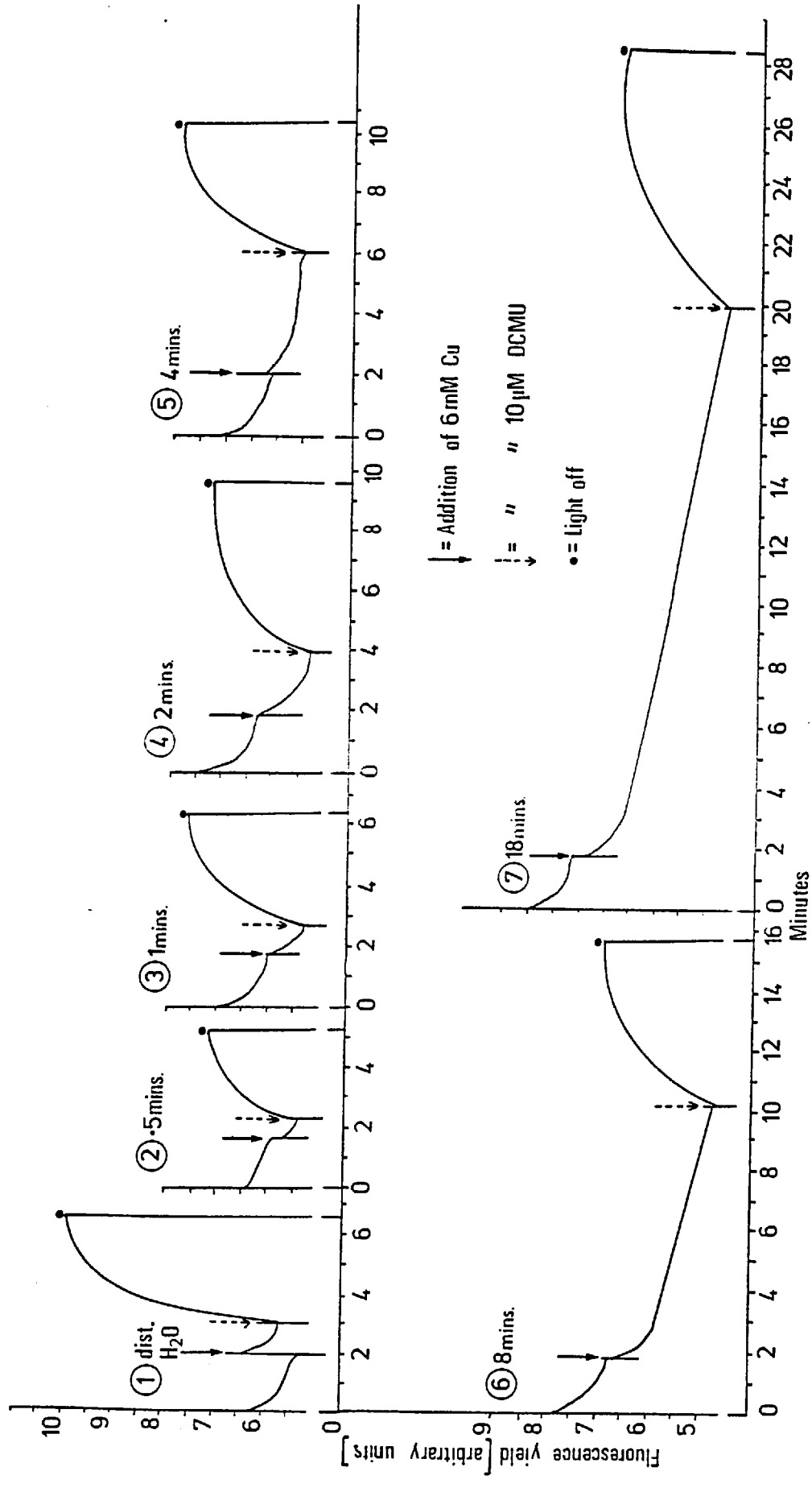


Figure 47. The effects on fluorescence yield of preincubating Chlorella cells with 6 mM copper for a range of different times, and of then adding DCMU.

TABLE XXI Effectivity of hydroxylamine as a fluorescence restorant following a metal treatment.

Copper treatment (mM)	Additions	Fluorescence yield after additions as % of initial control levels.
0	DCMU	142
0	DCMU + HA	134
2	DCMU	132
2	DCMU + HA	145
6	DCMU	114
6	DCMU + HA	140

The results show that, in the control situation, the presence of hydroxylamine slightly reduced the amount of fluorescence stimulation brought about by DCMU addition. With copper present, however, a noticeable extra stimulation occured when HA was present. It is of note, however, that, as illustrated by the 6 mM trace of figure 48, this extra fluorescence rise soon decayed away, and the increase was not maintained as it was with DCMU alone being present.

An identical follow-up experiment utilised the same proceedures, but lower copper treatments. The results, given as table XXII, are broadly consistent with those given above.

TABLE XXII The effectivity of hydroxylamine as a fluorescence restorant in Chlorella cells pretreated with low levels of copper.

Treatment (mM Cu)	Additions	Fluorescence yield after additions as a % of initial control levels
0	DCMU	166
0	DCMU + HA	160
0.159	DCMU	166
0.159	DCMU + HA	160
0.500	DCMU	150
0.500	DCMU + HA	157

At control and 0.159 mM copper levels, the addition of DCMU with HA resulted in a fluorescence stimulation slightly less than that gained with DCMU alone. At 0.5 mM Cu (and above - see Table XXI), however, the hydroxylamine gave rise to an extra fluorescence boost over and above that obtained with DCMU alone.

Experiments essentially the same as those outlined above were conducted using the donor system of phenylenediamine plus ascorbate. Table XXIII gives a

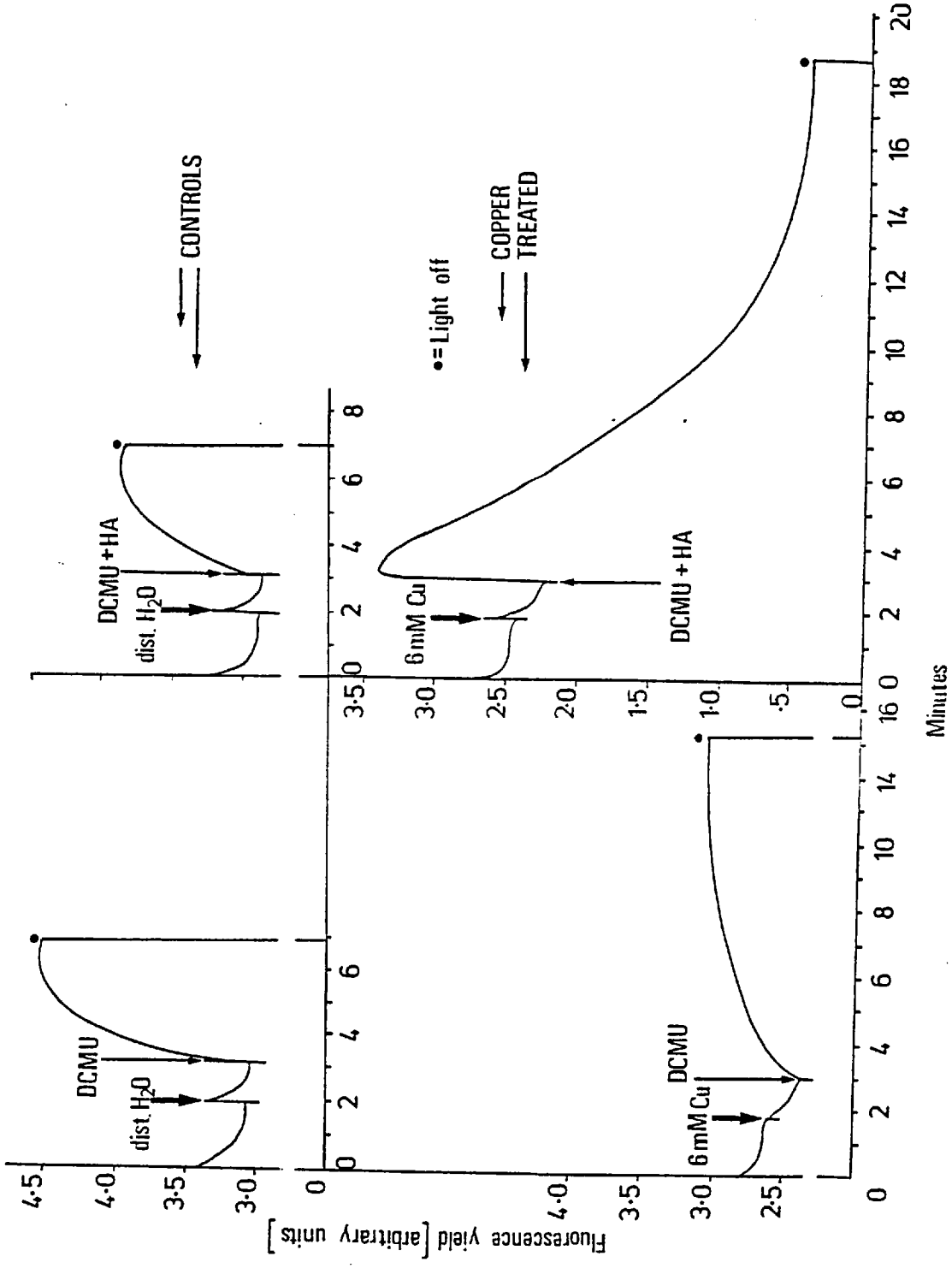


Figure 48. A comparison of the effects of DCMU and DCMU plus hydroxylamine on the fluorescence yield in controls and Chlorella cells treated with 6 mM copper for one minute.

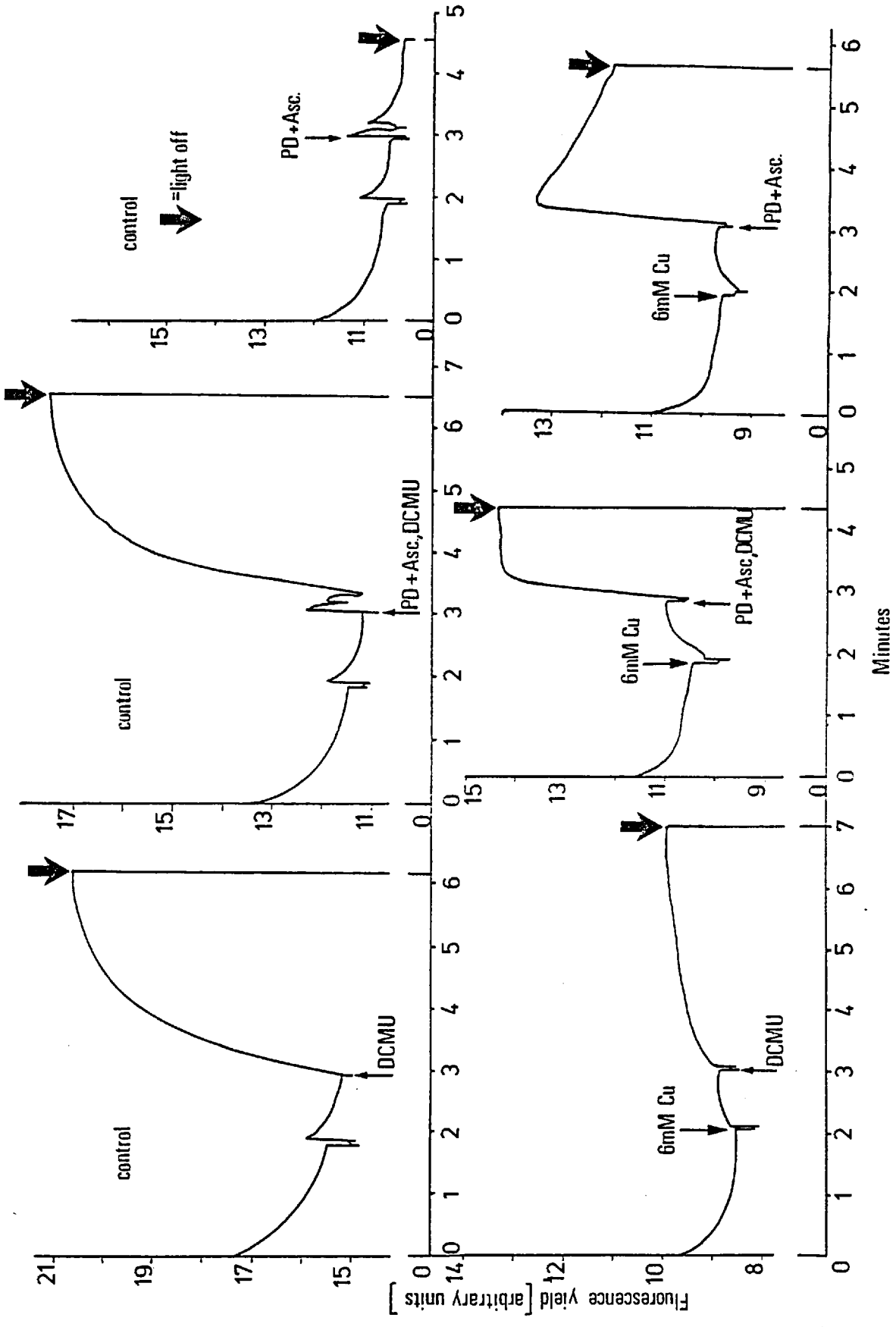


Figure 49. A comparison of the effects of DCMU and DCMU plus phenylenediamine with ascorbate on the fluorescence yield in control Chlorella cells and cells pretreated with 6 mM copper for one minute.

summary of the results that emerged from this work.

TABLE XXIII Effectivity of phenylenediamine plus ascorbate as a fluorescence restorant in Chlorella cells pretreated with copper. (All results are expressed as a percentage of initial mean control levels).

Copper treatment (mM)	Final fluorescence yield after the following additions. DCMU	PD + Asc.	PD + Asc., DCMU
0	145	97	155
0.533	148	146	149
6.000	122	139	138

The traces for 0 and 6 mM copper can be seen as figure 49. The results show that the 6 mM copper treatment severely reduced the fluorescence stimulation brought about by DCMU addition, but that the additional presence of the donor system resulted in a considerable fluorescence rise in the treated cells. The injection of PD + Asc. alone (no DCMU) into controls had no effect on fluorescence yield (beyond a slight apparent supression), while it gave a large fluorescence boost to copper-treated algae. However, once again, the high fluorescence yields obtained soon began to decay away.

CHAPTER IV

DISCUSSION

DISCUSSION.

The purpose of the Introduction section of this thesis was to give an idea of the range of approaches being adopted to study the toxicological action of copper on the functioning and growth of photosynthetic organisms. Also, the review attempted to show the relevance and importance of this metal in the context of environmental contamination and pollution. With regard to the experimental work of this thesis, particular emphasis was placed on aquatic plants, and a range of techniques used to try and discover the biochemically-important sites most sensitive to interference by copper. By trying to obtain a more detailed picture of the actual mechanisms of copper toxicology, and by narrowing down its sites of action, it was hoped that a contribution could be made towards an understanding of the toxicological importance of this metal in the environment. By deciding how copper acts, and at what levels, knowledge was accumulated in the hope of enabling potential polluters of the environment to foresee the likely outcome of a particular act of metal contamination.

The first section of this work, looking at growth and copper uptake, tries to give an overall view of the metal effects on the plant system as a whole. Using Chlorella cells, it was demonstrated that a graded growth response to increasing copper levels was readily obtained, a greater growth supression being evident with the highest metal doses. It was also shown that the threshold levels, beyond which growth retardation was apparent, varied somewhat according to the conditions prevaling. Thus, although low copper levels up to 2 or 3 ppm seemed to have no significant effects, while 4 ppm and above, in full culture medium, normally resulted in growth inhibition, the degree of the response varied with the concentration of the initial inoculum. This was a simple dilution effect with less metal being available per cell, or per sensitive site, when the cellular density was increased. Cultures growing under conditions containing up to approximately 5 ppm copper often showed that, after the initial inhibition, they had the capacity to recover somewhat, and even to begin to catch up with controls. This capability was only evident in cases where the early inhibition had not been too severe, and was probably the result of either the gradual loss of metal from solution as insoluble forms or due to the binding to insensitive sites on the algae, probably at the cell walls and slime envelopes. If the treatment is such that cell division is still able to continue, albeit at a reduced rate, then the amount of metal per organism will further decline with time.

In similar growth studies with the phaeophyte Sargassum muticum, results broadly in keeping with the above were obtained. In agreement with FLETCHER and FLETCHER (1975), this alga was found to grow best with 16 hours of daily illumination but, even under these conditions, copper treatments as low as 0.31 ppm seemed to completely prevent it from growing. A graded response to increasing metal levels was not achieved due to the extreme sensitivity of the plant.

By reference to the percentage of Sargassum tissues still respiring at the end of particular copper treatments, LD_{50} values of 0.22 ppm ($CUSO_4.5H_2O$, plus 4 days) and 0.24 ppm (release glass, plus 3 days) were obtained. The difference can be attributed to the slightly shorter experimental period used with R.G. These values can be compared with the 0.33 ppm copper (as the sulphate) quoted by LEWEY (1977). Sea water contains no chelators, or at least only low levels, and this might have served to increase the metal effects, giving the apparently high sensitivity found here. The observation that copper can exert severe toxicological effects on a multicellular system having a considerably more complex structure than Chlorella cells provides evidence to support the view that at least some extrapolations from results obtained with the unicellular system up to larger systems can realistically be made.

The work undertaken to monitor copper levels in solution during experiments, both with Chlorella and Sargassum, reinforced the results of the above growth studies because it was found that the concentrations were rarely as high as might have been expected from the additions made. This was mainly as a result of metal precipitation and loss from solution. McINTOSH (1975) had quoted a similar result for copper additions to natural waters. The overall point to be made therefore is that many of the calculated metal levels quoted (as opposed to measured by analysis) should, in fact, be revised downwards, and therefore the cells are even more sensitive to poisoning by copper than might have been supposed. This point is emphasised by the plot, shown as Figure 4, in which the average growth deviation of copper-treated cultures from controls, compared with the mean analysed copper concentration of the medium over the experimental period, resulted in a straight line, i.e. at least over the range of about 1 to 15 ppm external copper, there is a direct relationship between growth inhibition and medium copper content. However, the level of copper in the medium must, to some extent, vary with the total copper present in the system, and

therefore the same sort of relationship might be expected between growth inhibition and total heavy metal.

The experiments designed to investigate copper toxicity with various levels of chelator present, or in its complete absence, provided very convincing evidence that the presence of a non-living complexing agent reduced metal toxicity. Thus, it was demonstrated that, although it was in the cultures with chelator present that the levels of the metal were generally highest, lower concentrations, acting in tandem with a lack of chelator, gave the most serious growth inhibition. It was shown that it was possible to gain a graded response, whereby a given copper dose could result in increasing inhibition as the EDTA presence declined from the normal (0.27 mM) to, say, 0.001 mM. This gradually-increasing toxicity was more pronounced where the copper treatments were kept reasonably low (up to 5 or 6 ppm) so that no threshold effects seemed to be operating. A further experiment, in which cultures were grown with and without copper and with either normal or 1/10th normal micronutrient (and therefore reduced EDTA) levels, reinforced this point. It was shown that, although the two controls had identical growth patterns, only 1.49 ppm copper in the 1/10th normal samples was significantly inhibitory, while even 4.94 ppm had no effect on growth if the micronutrient presence was as usual.

Several other workers have quoted similar effects to the above with various species, used under a wide range of conditions. AUSTENFELD (1979), working with Phaseolus, had shown that the presence of EDTA reduced nickel uptake into the plants, and DeKOCK and MITCHELL (1957) demonstrated the same phenomenon for Ni, Zn and Cu in mustard and tomato. FOY et al (1978) agreed that more metal can be taken up by plants when it is in the ionic form than when chelated, and MARQUENIE van der WERFF (1979) took the work further by reporting similar results, but in the context of natural soil complexing agents such as humic acid. GACHTER et al (1973) had said that natural waters have the capability to mask added Cu^{2+} ions, this being attributed to ligands forming copper complexes. The toxicity of copper has therefore been shown to be related directly to the activities of cationic Cu^{2+}, CuOH and $Cu_2(OH)_2^{2+}$ (ANDREW et al, 1977). This is all in agreement with my findings; copper precipitates and complexes are, on the whole, non-toxicological. FITZGERALD AND FAUST (1963) even said that "approximately each part of EDTA in a medium can neutralise the toxicity of one part of copper sulphate" to an algal culture. It is, in some cases, debatable as to whether these results are the outcome of competition between the chelator and the

metal for binding sites at the plant surface (or at some location within the plasmalemma) or perhaps the metal-chelator complex has a configuration, or physical size, that is simply incompatible with the normal points of attachment or entry to the cell. NAKAJIMA et al (1977) have stated that, in the Chlorella, there are at least two binding states for copper. They said that one of these has only weakly-coupled ligands and could be easily substituted with EDTA. COOMBES et al (1978) reported results where it was shown that more copper could subsequently be desorbed from barley roots if the metal was originally applied as $(Cu^{II} (OH_2) 6)^{2+}$ than as either of the complexes $(Cu^{II} (en)_2)^2$ or $(Cu^{II} (gly)_2)^0$.

Whichever of the above mechanisms is the relevant one vis-a-vis my results, it is clear that EDTA is alleviating copper's effects by reducing the amount of metal that is reaching sensitive intracellular sites-either by competing for the ligands normally involved or by combining with the metal to give a form less available for plant uptake and adsorption.

That metal complexes are less toxic than other forms has perhaps been most dramatically underlined by the work of NAIKI et al (1957, 1957 and 1976) who have reported that yeast cells can detoxify copper ions by complexing them internally with either sulphur substances, nitrogenous compounds or proteins. SILVERBERG et al (1976), working with Chlorella cells, found that they could form nuclear complexes containing copper and this, they suggested, was also a detoxifying mechanism.

The points brought out by my work, taken in association with these other reports, have therefore established that it is non-complexed copper forms that are of most importance in toxicity studies. Total copper levels present are probably only relevant in so far as they will be a factor in determining the equilibria that govern the levels of readily-available metal in the system.

When the effects of varying the time of exposure to a particular metal treatment were investigated, the results obtained did not demonstrate clearly-defined progressions. However, trends were visible; figure 6 illustrated that up to 240 minutes exposure to copper levels as high as 40 ppm had a slight inhibitory effect on subsequent growth, and this trend was also seen in a similar experiment with 24.8 ppm but no EDTA wash after the incubation with metal (Table 5). Perhaps the most convincing demonstration of an increased period of exposure causing greater inhibition was obtained when the

cells were cultured after their use in oxygen electrode experiments (Figure 22). Here, using only a short exposure time, 5 and 50 ppm gave increasingly great growth inhibition. This result probably underlines the reason for the small scale of the effects noted in previous work; the oxygen electrode medium was a bicarbonate buffer and contained no chelator which was present, as normal, in the other experiments. This EDTA can, as demonstrated, drastically reduce copper's effectivity to act as a toxin. It seems to have been shown, therefore, that copper, even when present for only a relatively short time (about 15 minutes in the case of the oxygen electrode-derived cells), can exert an influence that will cause a reduction in the growth rate of the cells when they are subsequently transferred to fresh medium. This growth retardation, which seems to be reversible, at least over the time scales used here, is much less marked where EDTA is present during the metal incubation. The results seem to reinforce the idea that copper's preliminary effects on growth can be apparent early on; the metal must be able to reach sensitive sites quickly or, alternatively, it may be bound at the cell wall and only subsequently either migrate inwards to cause biochemical interference or, by staying in place, block the passage of metabolites.

The studies to follow the build-up of copper levels in solution caused by release glasses aimed to provide background information concerning the sort of doses that would be relevant for subsequent toxicological studies. It was basically shown that metal levels normally rose quite quickly in the initial release phases then declined somewhat. Chlorella medium was capable of achieving, and sustaining, levels of 10 or 15 ppm if RG doses of at least 2g/litre were used over periods of 10 days plus. The concentrations achieved in sea water were generally a little lower. The presence of chelator in the medium seemed to enable higher levels of metal to be sustained than when it was absent (Figure 9); the chelator would bind with copper ions and keep them in solution, thereby preventing them from linking to other groups that might normally result in the precipitation of insoluble forms. This point was underlined by a further experiment (Figure 10) with full medium and medium minus both EDTA and micronutrients. Here, the copper concentrations in the latter medium lagged even further behind controls, presumably because, with no micronutrients present for binding, even more copper ions could become linked to groups to form insoluble forms. It was also demonstrated to be the case that higher copper levels were evident in media containing cells than when it was Chlorella-free. (Figure 11 and 12). The differences in metal concentration became

more pronounced with time, and it may be that this was because, in the early phases, the cells were absorbing and adsorbing copper ions and there was little real difference in the levels in the media. However, the cells were providing a strong sink for the metal, maintaining a stimulus for it to be released at a faster rate, although, early on, very little, if any, of the extra copper would remain in solution. However, as time progressed, the metal would gradually exert adverse effects on the cells, leading to cellular degeneration (over a period of perhaps several days), and resulting in a release of the extra metal to the medium, thereby causing a general rise in the background level.

The first complete growth experiment conducted using a release glass demonstrated no real differences between development patterns of treated and untreated cultures, despite the fact that, in the case of the highest RG dose used, copper levels in the medium rose to nearly 15 ppm. This surprising result cannot be explained except by supposing that the initial cell inoculum (0.05% pcv) was sufficiently high for the cells to be able to cope with the prevailing metal levels. Then, as they continued to divide, they could largely manage to keep up with the gradually-rising metal concentrations. STEEMANN-NIELSEN et al (1969) have said that, on dividing, Chlorella forms "autospores" within the old cell and that when these seperate, the old cell wall and cytoplasmic membrane are cast off and lost. It has been shown to be likely that both of these structures bind copper and this may be a significant way of reducing copper levels in individual cells. The lower RG doses gave rise to copper concentrations that never went above 5 ppm and, with a chelator present, the cells could readily cope with this amount. The concentration of copper within the cells at the end of the experiment (Figure 15) followed an increasing trend with higher RG additions. The cells were apparently still capable of growth even when they contained perhaps several hundred microg. of copper per g (ppm) of cell ^{fresh} weight.

A further RG growth experiment summarised as Table 6, also failed to demonstrate any drastic growth supressions, and it was only when a higher range of RG doses were employed (Figure 16) that a graded response to increasing levels of addition was seen. However, even then the cells were still growing, albeit more slowly.

There was an element of variability in the release glass results, but it gradually emerged that for this form of application to be effective as a serious growth retardant,

it must either be present at reasonably high levels (at least 1.6g RG/200 mls) or the initial inoculum must be low. That the chelator presence was again drastically affecting copper's effectiveness as a toxin when supplied as a RG was demonstrated by varying the levels of EDTA in the media (Figure 17). It was shown that, by reducing the amount of EDTA in solution, a greatly increased growth supression was obtained which reached very high levels with only 4.8 ppm copper when the EDTA level was down to 0.01 mM.

When the work to study copper uptake into algae was begun, one of the first experiments conducted looked at Chlorella effects on medium pH. It was thought that the pH rise noted in inoculated media was probably occurring because the cells, during photosynthesis, were removing carbonate ions from solution, thereby causing a fall in the carbonic acid level, as well as reducing the carbonate/bicarbonate buffering capacity of the medium. Formation of the bicarbonate would also reduce the concentration of hydrogen ions H^+ (LUTON, 1980). Hydrogen ion binding by the cells is also possible (STEEMANN-NIELSEN and KAMP-NIELSEN, 1970) and this might contribute to the pH rise. When the cultures were stirred and vigourously aerated, the pH increase was reduced as the carbonate level in the medium would be constantly replenished.

This brief consideration of Chlorella-mediated pH effects was borne in mind in the studies that aimed to look indirectly at copper uptake by monitoring possible changes in copper concentration in the bulk medium. However, even when the appropriate controls enabled the pH and possible precipitation effects to be allowed for, no consistently-significant results could be obtained; experiments were conducted both with and without chelator being present and with different initial cell inocula, but definite trends did not emerge. The method, being indirect, had intrinsic problems associated with it; perhaps the cellular concentrations used were too low and the experimental periods too short. This would have meant that the amount of metal removed from solution was very small when compared to that remaining.

When cell copper contents were then assessed directly, in association with total and medium metal levels, using methods of cell digestion, a clearer picture emerged and results were more as might have been expected (Figure 19). Using copper sulphate as the metal source, the copper concentrations in the media were found to decline with time as the levels rose in the cells. As expected, the degree of growth inhibition

obtained followed the trend in increasing metal levels. The amount of metal accumulated by the end of the experiment, in those cells from the highest treatments, appeared very high (over 3000 ppm), but the results are compatible with those reported by other authors. For example, HASSALL (1963) quoted 5200 ppm metal in Chlorella vulgaris (dry weight basis) after 5 hours of contact with 8.3×10^{-4}M (approximately 52 ppm) copper, while HORIKOSHI et al (1977) mentioned 1250 and 2793 ppm for normal and scalded Chlorella regularis cells respectively after 4 hours of contact with 2.01 ppm copper. Other workers (KNAUSS and PORTER, 1954, McBRIEN and HASSALL, 1967, NAKAJIMA et al, 1977 and SAKAGUCHI et al, 1977) have given various values ranging from a few hundred to a few thousand ppm of copper in the cells. The values obviously vary widely with the experimental conditions, such as time of exposure, metal levels, concentration of cell inoculum, species involved etc.

Similar uptake experiments to the above were conducted using RG as the metal source (see Figure 20), and broadly comparable results were obtained. Total copper levels were found to rise only in those treatments where RG additions had been made. The same was true for medium copper concentrations; these were lower than those of the previous experiment reported above (Figure 19) and this, in turn, coupled with a higher initial cell inoculum (0.23% pcv was used in attempt to bulk up the samples for weighing) resulted in generally lower copper concentrations within the cells. Considering some of the results reported above, the growth supression visible for the RG-treated cultures was surprisingly high on this occasion, but the extent of the inhibition may have been exaggerated as the method used for the growth assessment in this case (the weighing of cells in culture aliquots) was rather crude.

In the work with Sargassum, the tissues seemed to have a high affinity for the copper and, on digestion at plus 15 days, a good straight line relationship was demonstrated, as with Chlorella cells, between copper in tissue and that in solution (Figure 24). As the segments had ceased growing after only a day or two, it seemed likely that the Sargassum tissues had been binding with metal even after their death.

The idea that dead botanical tissue acts as a metal sink was also demonstrated by following copper uptake by normal and scalded (i.e. dead) Chlorella cells. This work is of relevance in the context of the use of copper as an algicide as, if dead plant material can still bind with the metal and render it unavailable, then

there is an increased chance that cells that have survived will be able to continue to grow and divide. My results showed that not only could scalded cells continue to bind copper, but they were also capable of binding more of it than could normal cultures. The use of lower initial inocula resulted in the cells containing more copper than when the inocula were higher. HORIKOSHI et al (1977) had reported similar results, and said that not only could scalded cells take up more copper, but they could also do so more quickly. These workers also reported that 70% of the copper taken into scalded cells was released by an EDTA wash, while only 17 to 19% came off from normal cells under the same conditions. This evidence led them to conclude that "the distribution of the copper in the scalded Chlorella cells was different from that in the living cells".

The overall point that algal cells can act as very efficient sinks for heavy metals was perhaps made by JENNETT et al (1979) when they said that algal meander systems have proved to be 99% efficient at removing heavy metals flowing out of mine tailings.

When copper's effects on Chlorella photosynthesis and respiration were assessed in an oxygen electrode, it was found that photosynthesis was rapidly inhibited by all of the treatments used. However, respiration was found to be stimulated by up to 75% by copper levels as high as 40 ppm. This result was at first surprising and it is difficult to imagine this situation continuing in the longer term. GREENFIELD (1942) had found 50% photosynthetic inhibition in Chlorella cells subjected to 5 μM copper sulphate, a very low level. GROSS et al (1970) also reported respiratory inhibition by low copper concentrations, and McBRIEN and HASSALL (1967) had said that both respiration and photosynthesis were severely affected when the uptake had occurred under anaerobic conditions. This, they said, was probably due to copper becoming bound to sites, perhaps sulphydryl groups, not normally available to the metal. HASSALL (1963) had formed similar conclusions, but was uncertain as to whether the higher toxicity of the metal under anaerobiosis was due to a change in the chemical nature of the applied copper or to the formation of complexes. However, more in keeping with my results concerning cell respiration, CEDENO-MALDONADO and SWADER (1974) had observed a stimulation of photosynthesis and respiration over the first few minutes following a treatment. This was true for treatments of up to 1000 μM copper (i.e. higher than those that I used), and they conjectured that this might have been due to the cupric ions causing an increase in the permeability of organelle membranes, resulting in an increase in the availability of reaction substrates.

Inhibition of photosynthetic electron transport was more clear-cut than that shown for respiration and no trend was demonstrated for stimulation occurring. Several authors have reported the same sort of inhibition with a range of different metals, including copper, and also in the case of algal species other than Chlorella. (De FILIPPIS and PALLAGHY, 1976, HOSTETTE, 1973, SARGENT and TAYLOR, 1971). STEEMANN-NIELSEN et al (1971) argued that it is the accumulation of copper on the cytoplasmic membranes that inhibits photosynthesis by causing the build-up of assimilation products. They further concluded that metal penetration into the plasma was not important. In the light of the results obtained in some of my experiments that investigated copper's effects on partial electron transport reactions, this statement may perhaps be questioned to some extent. It is true that, in an intact Chlorella cell, there will be a progression of effects whereby the most readily-available sites are affected or inhibited first. Thus, it is possible that the build-up of metal ions at the cell surface might be among the first responses to a copper treatment, but it seems likely that penetration would then rapidly occur and interference and inhibition on a more biochemical level would soon become apparent.

The above brief series of experiments with the oxygen electrode were followed by the studies to look at copper's effects on chlorophyll levels and chlorophyll a:b ratios in Chlorella cells. The results indicated that, over the time periods investigated, metal concentrations of about 18 ppm and above were required to give a significant difference from controls. Declines in both total chlorophyll levels and a:b ratios could be seen after the use of this sort of treatment. The decline in the ratios indicates a reduction in the amount of chlorophyll a present in the cells. GROSS et al (1970) had quoted that copper can cause a reduction in the total pigments of Chlorella cells concurrent with photosynthetic inhibition. De FILIPPIS and PALLAGHY (1976) reported similar results obtained after a mercury treatment. They put forward the suggestion that "mercury initially enhances the oxidative steps and inhibits the reductive steps in the synthetic pathway of these pigments". BASZYNSKI et al (1980) grew tomato plants on nutrient media containing cadmium and found that, attendant upon reduced nett photosynthesis rates, there was also a noticeable fall in the levels of chlorophyll and accessory pigments. After 14 days in media containing 20 μM $CdSO_4.8H_2O$, the chlorophyll a and b content of the leaf tissue was about 52% reduced, compared to controls, and the a:b ratio fell by approximately 15%. The time scale of the responses observed in my results are such that it is difficult to imagine changes in pigment levels as being a primary cause of photosynthetic inhibition in

Chlorella cells. It is not known if this is a result of the insensitivity of the pigment-assessment techniques but, nonetheless, it does seem likely that one way that, in the longer term, metal ions, and perhaps especially copper, can adversely affect the rate of photosynthesis is by causing these changes in pigment concentration.

In the electron transport work undertaken with isolated chloroplasts, amongst the first experiments were studies to look at the effects of SO_4^{2-} ion on the rate of electron flow to methyl viologen. The results confirmed that, at the levels present in subsequent experiments, the sulphate ion was only making a small difference to electron transport as compared with control rates. Uncoupled electron transport was more affected by sulphate treatments than was coupled, but in both cases there seemed to be no real progressive decline in rates with time because of the SO_4^{2-} (in agreement with the result of CEDENO-MALDONADO and SWADER, 1974).

The range of 0 to 16 ppm copper treatments caused increasingly severe inhibition of electron transport to methyl viologen in uncoupled chloroplasts. Even 1 ppm copper caused a significant inhibition of rates. CEDENO-MALDONADO et al (1972) had also found that Cu^{2+} ions could inhibit uncoupled electron transport, to NADP in this case, at very low concentrations. The picture was less clear for coupled rates where levels of 8 ppm and above seemed to be inhibiting slightly less than were lower concentrations. This was the first indication obtained that copper might be capable of acting as an uncoupler of photophosphorylation.

In the preincubation experiments, in which the chloroplasts were kept on ice in various copper treatments and then periodically sampled, the same broad trends as the above were demonstrated; the chloroplasts were slightly more sensitive when uncoupled than when not, but there was a graded response in both sets of results, whereby higher copper concentrations caused greater inhibition. A point of note was that the bulk of the response to a given metal treatment was often detectable right from the "time zero" sample and there was, in those cases, then only a relatively minor further decline in rates with time. This was generally less true for the lower levels of copper addition than for the higher, however. An anomalous stimulation of uncoupled rates by a 2 ppm copper treatment was observed on one occasion (Figure 28); this can only be explained by assuming that the NH_4Cl had not entirely effected the uncoupling of ATP formation and therefore the copper might have acted

to complete the process, causing the usual increase in electron transport rates.

When the effects of preincubation with copper in the light and the dark were compared, it was found that the dark pretreatment decreased the inhibitory effects with respect to controls. Uncoupled rates were again repressed according to the level of metal presence, and to the length of the preincubation period. Coupled rates were also similarly affected by increasing copper concentrations, but over the experimental period there was a trend for the results to actually increase in some cases. This was taken as further evidence that copper was acting as an uncoupler. This uncoupling action was more obvious either when the copper treatment was sufficiently low that its associated blocking effects on electron transport were relatively slight, or when the effects of a higher dose had been somewhat reduced by a dark preincubation.

The results therefore suggested that, in the light, sensitive sites are more available for disruption than they are in the dark. This may be brought about by conformational changes in membrane arrangement, whereby more sensitive sites become exposed in the light. This rationale is in agreement with CEDENO-MALDONADO et al (1972) who reported a reduced inhibition after a copper treatment in the dark or with lower light intensities. In a later paper (1974), CEDENO-MALDONADO and SWADER reported that, in Chlorella, resistance to inhibition of photosynthesis by copper "increased slowly but steadily by increasing period of dark". GREENFIELD(1942) and STEEMANN-NIELSEN et al (1969) had also reported broadly consistent results.

These experiments designed to investigate the action of copper on both photosystems (PS I and PS II) had therefore shown that:

(1) Copper inhibits both coupled and uncoupled rates of electron transport.
(2) There is a graded response, whereby more metal causes a greater inhibition.
(3) A high proportion of the inhibition is exerted very rapidly, almost instantaneously, and therefore, in broken chloroplasts, the sensitive sites on the thylakoid membranes must be freely available
(4) Uncoupled rates are slightly more sensitive to inhibition than are coupled.
(5) Pretreatment of broken chloroplasts with copper in the dark instead of the light results in reduced inhibition of rates and

(6) In coupled chloroplasts, the copper seems to be able to exert an uncoupling action in tandem with the normal inhibition. This uncoupling effect seems to be maximal either when relatively low copper concentrations were used (up to about 5 ppm, 79 μM), or when the associated inhibition, caused by a higher level, was reduced by dark pretreatment.

When PS I of isolated chloroplasts was assayed seperately for sensitivity to interference by copper, the results fitted in neatly with those previously obtained for the whole electron transport chain. Uncoupled rates were again more seriously affected than coupled. The traces produced, showing the effects of various copper concentrations on PS I uncoupled rates over time (Figure 30) , followed a very neat progression with higher levels causing more inhibition and with a decline as the time of preincubation increased. However, with coupled chloroplasts the results were more confusing due to copper's dual action as an inhibitor of PS I electron transport and as an uncoupling agent.

This inhibition of PS I transport rates is consistent with the results of SHIOI et al (1978a) who said that copper interfered with the activity of ferredoxin and reduced $NADP^+$ photoreduction. This observation could explain my findings, and the scale of the inhibition was also shown by CEDENO-MALDONADO et al (1972) and OVERNELL (1975) who demonstrated that only 1 or 2 ppm could signigicantly inhibit PS I transport rates.

Studies were made to compare the effects of a fixed copper dose (5 ppm, 79 μM) on a range of chloroplast (i.e. chlorophyll) concentrations in an effort to further elucidate the apparent uncoupling action of copper. The results for uncoupled rates seemed to simply demonstrate a dilution effect as, although all of the rates fell behind those of the controls, there was less inhibition when more chloroplasts were present. Similar results had been found by STEEMANN-NIELSEN et al (1969) and by CEDENO-MALDONADO et al (1972 and 1974), and indicated that the degree of damage was proportional to the amount of metal bound to the membranes. When coupled rates were examined, however, the trend was the opposite; all of the rates were above control levels and the greatest stimulation occurred with the lowest chlorophyll concentrations. This, taken along with Table 12, showing how the ratios of coupled:uncoupled rates in the different treatments varied over the experimental period, was further evidence

that copper can induce uncoupling. With 25 µg chlorophyll/ml in the reaction mixture, ratios of coupled to uncoupled rates (both expressed relative to controls) rose from 1.53 to 3.89 over the 32 minute preincubation period, indicating that there had been progressive uncoupling. With 100 µg chlorophyll/ml. the values only ranged from 1.32 to 1.64, a far smaller increase.

When PS II was investigated, the trends discovered were again similar and compatible with the previous arguments. Preincubation experiments, involving up to 20 minutes of treatment in copper concentrations of up to 30 ppm, indicated again that, although all of the samples were inhibited with respect to controls, there was a slight stimulation of some coupled rates over the experimental period (Table 13). Thus, at time zero, the 1 ppm Cu-treated coupled rates were 89% of controls, but they rose to 94% at plus 20 minutes. This trend for gradual uncoupling was only really apparent at low metal concentrations; with 4, 5, 6 and 7 ppm copper treatments, the copper levels seem to have been sufficiently high for any uncoupling to become apparent by the time zero reading, and therefore the degree of the initial inhibition appeared to be supressed. Thereafter, the rates declined as the metal did more and more damage. At copper levels of 10 and 30 ppm, the treatments were becoming sufficiently high for this trend to be reversed again, i.e. although there was sufficient metal present to cause a high degree of uncoupling, the level was such that the inhibition of electron flow was already severe.

In contrast to the above, the associated uncoupled data (Figure 33) showed a more ordered progression of inhibition, as also previously demonstrated with PS I work. With broken chloroplasts, uncoupled with NH_4Cl, there was no evidence for copper causing further uncoupling and all of the traces illustrated a decline in rates with time.

My experiments to look at copper's effects on the 518 nm absorbance change of isolated chloroplasts, following the techniques of OLSEN et al (1980) and TELFER at al (1980), provided further evidence for the uncoupling action. It was found that the presence of copper increased the rate of decay of the absorption peak at 518 nm. The results were broadly similar for the three copper concentrations used (3.33, 10 and 20 ppm), indicating that, under the conditions employed, a threshold effect was operating. This Cu-induced decrease in the $T_{\frac{1}{2}}$ values for the decay phase of the absorption traces was an indication that the chloroplasts were becoming progressively

more uncoupled, as the decline occurs as a result of the membranes becoming more permeable to H^+ and cations. The peak of absorption at 518 nm is dependant on the charge seperation at the thylakoid membranes that is a result of electron flow through the photosynthetic chain. The electrical field associated with the electrical gradient causes slight changes in the molecular configuration of the bulk pigments present. An uncoupler will facil itate the dissipation of the electrical gradient and therefore precipitate the decay of the absorption peak. Photophosphorylation depends on the existence of the electrical gradient and so evidence of a decline in the charge seperation is also evidence for a decrease in the degree of coupling of the thylakoids. My results all showed that the $T_{\frac{1}{2}}$ values fell progressively with time. This was a clear indication that the copper was exerting its effects gradually with more uncoupling as time passed.

The general inhibition of electron transport within PS II by heavy metals has been noted in several publications and both algae and isolated chloroplasts have been used. Thus, OVERNELL (1975) and SHIOI et al (1978b), working with broken Chlamydomonas reinhardii cells and Ankistrodesmus falcatus respectively, both reported PS II inhibition by cupric ions. SHIOI et al found 45% inhibition of the DCIP Hill reaction after 20 minutes preincubation with 10 μM (<1 ppm) $CuSO_4$. Other metals such as lead (MILES et al, 1972), zinc (HAMPP et al, 1976) and cadmium (BASZYNSKI et al, 1980, LI and MILES, 1975) have also all been shown to interfere with PS II in isolated chloroplasts. Working with copper and using various electron donation systems, CEDENO-MALDONADO et al (1972 and 1974) developed the theory that copper was acting somewhere on the oxidising side of PS II. SHIOI et al (1978) agreed with this idea; they found that diphenyl carbazide, donating electrons between the water-splitting act and the PS II reaction centre, could not restore transport rates after copper inhibition, and therefore the metal was acting at a point after the site of donation. A plot of the mean transport rates obtained over the experimental period with a variety of metal levels present showed that uncoupled chloroplasts, with respect to controls, were more sensitive to inhibition than were coupled. DUIJVENDIJK-MATTEOLI and DESMET (1975) had similarly reported an inhibition of uncoupled rates, but in their case by cadmium. These results were of particular interest because it was found that, when using methyl viologen as an electron acceptor, with coupled chloroplasts, a double effect of cadmium was obvious over the range of 1 to 10 mM (high levels compared with my experiments). Low cadmium concentrations brought about an increase of oxygen consumption which could be explained by an uncoupling of the photophosphorylation, resulting in an increase in the

electron transport. It was reported that 4 mM cadmium gave the maximum amount of uncoupling and that higher levels only resulted in inhibition of the rates. Furthermore, the work also went on to show that when the chloroplasts were preincubated with a known uncoupler (20 mM CH_3NH_3Cl in this case), "only the inhibitory effect of cadmium was observed".

JACOBS et al (1956), working with animal mitochondria, had also reported that the cadmium ion was a "powerful uncoupling agent" of phosphorylation, and that its effect was reversible. Amongst the compounds found to be able to completely reverse the inhibition was EDTA at 1 mM. It was also found that Cd^{2+} added to the mitochondria was bound very strongly and was not removed by repeated washing with sucrose. The authors put forward two possible explanations for the mechanism of the uncoupling action. Firstly, Cd^{2+} might displace another cation essential for phosphorylation from its active site. The second idea was that Cd^{2+} might block a free active site. An enzymic disulphide group was put forward as a potential candidate for the site of such a block. The data presented was insufficient for a choice to be made between these two possibilities. The paper went on to quote that the order of efficiency of uncoupling of the metals tested was $Cd^{2+} > Zn^{2+} > Cu^{2+}$.

MILLER et al (1973) also investigated cadmium's involvement with mitochondria, in this case isolated from corn plants, and found an inhibition of the oxidation of exogenous NADH at metal levels of 0.1 mM and above. Their data showed that dithiothreitol, a protector of SH groups, prevented any effect of cadmium on respiration. This indicated that "sulphydryl groups are likely involved in the cadmium-membrane reaction". They followed up their mitochondrial studies by noting that, as Cd^{2+} can be taken up by the roots of corn, concentrated throughout the plants and then become toxic even at extremely low treatment concentrations, then it is possible that the in-vitro cadmium effects on mitochondria, reported in the paper, were also relevant in the in-vivo situation.

De FILIPPIS (1979b) studied the effects of mercury and zinc on the growth of Chlorella cells and found that, amongst other chemicals, sulphydryl (-SH)-containing compounds, including the amino acid cysteine, could somewhat alleviate the toxicological effects of the former metal, although not of zinc. This was only true if the cells had been preincubated with the protective agent before the application of the mercury, and he surmised that the probable mechanism of detoxification was by the

-SH groups binding to the mercury in the medium and making it unavilable to the cells for uptake. Mercury has a relatively high association equilibrium constant with L-cysteline, for example, whereas zinc does not. Other compounds containing sulphur but having no -SH groups, provided no protection against either mercury or zinc. Another interesting observation was that cells incubated with sulphydryl group compounds contained greatly-elevated concentrations of sulphydryl groups within the cells. These elevated levels would be sufficient to bind much of the mercury entering the cell, "thus, reducing its freedom to be translocated to the sensitive parts". This work can therefore serve as a further illustration of the way that -SH groups are of obvious importance in the interactions between plant physiological mechanisms and heavy metal ions. If one artificially engineers conditions such that the number of sulphydryl groups within cells, or in their vicinity, is increased, then at least in the case of mercury, there will be an apparent reduction in subsequent metal toxicological effects.

LUCERO et al (1976) have reported that $CdCl_2$ can inhibit both cyclic and non-cyclic photophosphorylation in isolated spinach chloroplasts, and they also showed a depression in coupled electron transport. STRICKLANDand CHANEY (1979) looked at the influence of cadmium on respiratory gas exchange in Pinus resinosa pollen. They decided that an uncoupling action was not the metal's only method of interference, but that the influence on gas exchange was the result of "inhibition at multiple sites".

Thus, various groups of workers have looked at the involvement of cadmium with the coupling of phosphorylation to electron flow, whether it be in chloroplasts or in mitochondria. Evidence for a definite uncoupling capability of the metal has been put forward, and some authors have suggested that the site of action might be the sulphydryl groups of enzymes. The same depth of treatment does not seem to have been applied to copper in the literature, but some of the above points do provide good parallels for some of my results, obtained with cupric ions. The levels of metal that, for instance, DUIJVENDIJK-MATTEOLI and DESMET (1975) used (4 mM Cd) were considerably higher than those of my experiments (eg. 30 ppm copper is approximately equal to 0.47 mM); this must, to some extent at least, reflect the different relative toxicities of the two metals. In algal systems, copper seems to be more toxic than is cadmium, and this may help to explain why the uncoupling effects are less apparent at higher metal dose levels.

To what extent the same sort of possibilities for the mechanisms of uncoupling as those suggested for cadmium are relevant for copper is uncertain, but, remembering copper's high potential reactivity with biological ligands, it seems likely that interference with sulphydryl linkages is at least one possible explanation.

When preincubation experiments with PS II-active chloroplasts in the light and the dark were carried out, a definite trend emerged for light to increase the effectivity of copper to act as a metabolic poison, although the partial dark protection was lost at higher metal levels. This result was true for both coupled and uncoupled rates, indicating that light was not simply increasing the uptake of metal by means of an active, ATP-driven process. It might have been supposed that energy from photosynthetic electron transport would have increased the passage of cupric ions through the grana stacks of the thylakoid membranes in isolated chloroplasts, but in uncoupled material, the presence of ammonium ions would have caused the cessation of photophosphorylation; no substrates for ATP formation were added and the endogenous levels of these compounds in isolated chloroplasts are low. It therefore seems much more likely that light increased the electron transport inhibition by causing some sort of membrane conformational change that stimulated the exposure of sites in the transport chain to which copper could bind. This supposition agrees well with CEDENO-MALDONADO and SWADER (1974) who reported that light increased the toxicity of cupric ions both in intact Chlorella cells and in isolated spinach chloroplasts that did not have intact outer envelopes i.e. "the light enhancement of cupric ion toxicity included more than an energy requirement for the movement of cupric ions through the plasmalemma and the outer chloroplast membranes".

When the effectivity of 5 ppm (79 μM) copper as a toxicological agent with three different chlorophyll concentrations was tried, as in the similar PS I experiments, the apparent uncoupling capability was again demonstrated. It was found that increasing the chlorophyll concentration caused a decrease in the inhibition of uncoupled chloroplasts, but a corresponding increase in the inhibition of those that were coupled. This again reflects the fact that, with more chloroplast membranes present for binding, there will be less copper per thylakoid and therefore less uncoupling will occur.

When comparisons of the susceptibilities of the two photosystems to inhibition by copper were made (Figure 36 and 37), it was clearly shown that PS II was the more

sensitive. Thus, copper levels of 1, 5 and 10 ppm inhibited both sets of samples, but PS I was more resistant to inhibition than was PS II. When the effects of 5 ppm copper on five different chlorophyll concentrations were investigated, the trend in the results was the same. This seemingly indicates that a particularly sensitive site, or sites, exists within the confines of the PS II pathway, as assessed, and that, as it is more sensitive to inhibition than is the corresponding PS I site, then it is likely to be one, at least, of the primary sites of action. Results obtained with different chloroplast preparations have given slightly different degrees of response to various treatments, but significant inhibition of PS II uncoupled rates by low levels (1 ppm, 16 μM) have been demonstrated.

The fluorescence work, which constituted the final phase of these studies, tried to elucidate further the PS II inhibition. The addition of increasing levels of copper and cadmium resulted in a progressively-severe inhibition of PS II fluorescence (Figure 40 and Table 15), showing that the metals were interfering with electron flow to the Q pool. This would reduce the degree of reduction of substance Q and cause the drop in fluorescence. Copper was the most severe inhibitor of the two, although whether this is a reflection of its success in blocking a particular site, or of action at different locations is unclear. HOMANN (1969) and SHIOI et al (1978) had reported PS II fluorescence quenching by copper, while MILES et al (1972), working with lead, had reported that its inhibitory site was between the primary electron donor of PS II and the site of water oxidation, this being demonstrated by the restoration, using hydroxylamine, of normal fluorescence following lead inhibition.

The presence of cations (5mM $MgCl_2$) seemed to slightly reduce the copper inhibition of fluorescence (Table 16). The differences were not dramatic, but may perhaps be explained by the membrane conformational changes brought about by cation presence. MURATA (1969) had reported that the addition of magnesium ions increased the yield of chlorophyll a fluorescence of pigment system 2 and decreased that of system 1, concluding that the ions supressed the excitation transfer from bulk chlorophyll a of PS II to that of PS I, i.e. the spill-over step of excitation. In 1970, MURATA et al followed this work up by saying that the changes in the efficiency of excitation transfer brought about by such cation additions could be explained by changes in the membrane structure of the chloroplast lamellae. This was in agreement with HOMANN (1969) who postulated that the main reason for the change in the light emission is a cation-induced structural change in the grana of the chloroplast. This change in the arrangement of the thylakoid membrane structure may therefore result in a lowering of the accessibility of sensitive sites, thereby reducing copper's inhibitory

effects. The increased cation presence will result in closer stacking of the thylakoids, and it is not difficult to surmise that this would render it more difficult for copper to exert its action in the short term. The same results were not demonstrated with cadmium, and this may reflect a difference in the mechanism of inhibitory action with the two metals.

A similar series of experiments to compare fluorescence inhibition with and without DCMU being present did not demonstrate any differences between the two sets of results. The presence of DCMU prevents the reoxidation of substance Q, reduced by electron flow through PS II (DUYSENS and SWEERS, 1963). The fact that DCMU was irrelevant to the magnitude of the response to copper shows that the effect of the metal is occuring before the DCMU block at a site completely seperate from it. A reading of the literature gives a view compatible with this result; other authors have pointed to the primary site of metal action as being early on in the PS II electron transport chain, i.e. well before the point of DCMU involvement.

Experiments showed that it was really only with tris-washed chloroplasts that significant fluorescence increase could be observed on the addition of various artificial electron donors such as hydroxylamine, manganous chloride, diphenylcarbazide and phenylenediamine with ascorbate (Figures 41 and 42). This presumably reflects the fact that, in normal chloroplasts, electron transport rates are such that the degree of reduction of the pool of substance Q is more or less maximal and cannot be further increased. In tris-treated chloroplasts, however, the treatment puts at least a partial block on the transport chain very near to the water-splitting act (HOMANN, 1969; BLANKENSHIP and SAUER, 1974), probably by removing manganese from the pathway, and therefore it is possible for fluorescence levels to be raised by artificial donors as their addition will merely contribute to the restoration of normal yields. The 70 to 80% fluorescence increase with tris-treated thylakoids on the addition of DCMU was at first surprising, but has been explained by YAMASHITA and BUTLER (1969) who suggested that there are "sufficient endogenous electron donors to photoreduce Q if the electron transport out of Q was blocked by DCMU". If the tris wash is indeed removing manganese from the chain, then the fluorescence increases brought about by the artificial donors show that they are all supplying electrons at a point after the point of tris involvement in the system.

Tris-treated chloroplast thylakoids appeared to be less sensitive to fluorescence inhibition by copper than were normal isolated membranes (Table 19). This is evidence that copper is exerting its influence at the same site at which tris acts or at a point after the tris-sensitive site so that its effect will be smaller as fewer electrons will be flowing through the system. However, the chloroplasts are still partially sensitive, and it seems likely that the metal is acting at a point after the tris block. This idea was borne out by the experiment (summarised as Figure 43) that investigated not only the effects of a range of copper concentrations on tris-treated thylakoids, but also looked at how the response to subsequent DCMU addition varied. Higher copper doses caused, as expected, increasingly great declines in fluorescence yield, but the fluorescence rise brought about by DCMU also fell as the metal treatment rose. This implies that PS II was metal sensitive at a point after the tris-sensitive point, i.e. at a location after the normal donation site of the endogenous donors mentioned by YAMASHITA and BUTLER (1969). By inhibiting after this site, copper would effectively prevent the rise normally seen when DCMU was added.

That the copper inhibition of chloroplast fluorescence was indeed a gradual process with a progressive build-up to toxic symptoms was under/lined by the time course sampling experiments involving preincubation of organelles in a range of metal concentrations (Figure 43). The gradual drop in fluorescence yields over time showed either that it took up to several minutes for copper to reach all of the sensitive sites or that over this time period, the metal was affecting fluorescence via secondary routes. Either of these could explain why the full effect of a particular dose was not evident straight away.

The electron donation system of phenylenediamine was found to be able to partially restore copper-inhibited fluorescence yields only when the metal treatment had been low (up to 1 ppm, 16 μM). Beyond this level, no increase was noticeable and, once concentrations of 3 or 4 ppm were exceeded, fluorescence yields continued to decline even when the donor was added. At the lower metal concentrations, the application of DCMU following donor addition could result in a further fluorescence increase, but this capability was also only evident up to about 1 ppm copper. These observations may be explained in one or two ways. It may be that, with a low copper treatment, not all of the sensitive sites were completely blocked or inhibited and therefore the unaffected ones could let electrons flow through the system from the

site before the point of copper inhibition. Alternatively, phenylenediamine and ascorbate might be donating electrons after the main point of copper damage, but the metal might also be interfering with other components of the chain further on, thereby preventing the donor from effecting complete fluorescence restoration.

It proved impossible to effect any degree of fluorescence restoration in copper-treated chloroplasts with either hydroxylamine of manganous chloride acting as the electron donor. MILES et al (1972) had shown that hydroxylamine could restore fluorescence after a lead treatment, and so the situation vis-a-vis copper (and cadmium) involvement with PS II must be different. It seems that copper was inhibiting at a point after the points of electron donation by these compounds. The tris wash removes manganese from the system, and this metal is believed to be involved at a point very early on in PS II, close to, or associated with, the water-splitting complex. For manganous chloride to effect fluorescence restoration therefore, copper would probably have to be acting at a point where electrons enter the system from water, and there is no evidence to suggest this.

When Chlorella cells were used in fluorescence experiments, higher metal concentrations had to be employed than with chloroplasts in order to gain any significant responses. This is because, in isolated chloroplasts, the sites of inhibition are much more exposed and available for metal interference than when there is a plasmalemma, cell wall etc. intervening. With control Chlorella cells, the addition of DCMU resulted in a fluorescence yield increase of from 80 to 90% (Figure 47 and 48), but this stimulation was reduced if the DCMU addition was preceeded by a copper treatment, the extent of the rise obtained depending directly on the metal concentration employed. This implies that, with intact Chlorella cells, the pool of substance Q is not fully reduced and that the addition of DCMU, effectively blocking electron flow from Q to PS I, enables a greater degree of reduction to be achieved and therefore results in higher fluorescence levels. If, however, a copper treatment is administered before the DCMU addition, then at least a proportion of the total electron transport through PS II to Q will be impeded and, therefore, even when DCMU is added, the flow of electrons, probably still decreasing in volume, will be insufficient to achieve the complete reduction of all of substance Q.

Not only the concentration of the applied metal, but also the time of exposure was found to be important to the magnitude of the inhibition obtained

(Figure 47). The longer were the cells incubated with the metal, the greater was the drop in fluorescence yield-and the smaller was the subsequent rise obtained when DCMU was then added. This is all indicative of a gradual penetration of the metal to sensitive sites within the cell, perhaps coupled with secondary effects, caused by the metal acting on other systems. Initially, much of the metal would be bound to insensitive sites at the cell wall, but it would also gradually be absorbed to biochemically-sensitive locations. The gradual development of inhibition was perhaps more noticeable than with isolated chloroplasts because of the relative complexity of the system.

Hydroxylamine was shown to be able to boost the fluorescence increase, relative to controls, gained on DCMU addition after a copper treatment; however, this was only the case if a metal treatment had been used. In the control situation, or with no DCMU present, hydroxylamine gave rise to no fluorescence increase. Very similar results were obtained with the phenylenediamine and ascorbate system. However, in both cases, the high fluorescence yields obtained were transient and they immediately began to decay away quite rapidly. The reason for this fluorescence quenching was unclear, but the results probably point to copper acting within the PS II electron transport chain of intact Chlorella cells at a point before that of electron donation by these two systems.

The work of this thesis has concentrated on the effect of copper in controlling algal growth and explored the mechanisms of its interactions with the components responsible for photosynthetic electron flow. Clearly, at the cellular level, the toxicological action of copper cannot be restricted to photo-induced electron flow even though this is such a key process to the survival of photosynthetic organisms. Presumably, the metal will interfere with a host of physical and chemical reactions critical to the well-being of a healthy biological cell. It does not seem unreasonable to assume that copper will interfere with processes such as mitochondrial electron flow, the synthesis of metabolites and biochemically-important structural and functional components such as proteins and nucleic acids respectively, and with vital transport processes which occur across membranes. No attempt has been made to give an overall review of all of the literature in these areas or to explore all of these possibilities, and certainly it would be incorrect to suggest that the studies presented in this thesis are exhaustive and complete. Nevertheless, some basic experiments have been carried out and new information obtained. It is hoped that in the future more detailed studies of the toxicological action of copper on photosynthetic material will be

conducted and that a thorough understanding of the processes involved elucidated both at the cellular and multicellular levels.

Also, during the course of my experimental work, it became clear to me that heavy metals, like copper, which have specific sites of interaction could prove to be important experimental tools for investigating the nature of a variety of cellular processes. In my own case, it seems to me that the copper inhibition of electron transport from water to the photosystem two reaction centre could be used as a means for investigating the processes which take place in the region of water oxidation, an area of immense importance, but still far from being understood.

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