J. mar. biol. Ass. U.K. (1976) 56, 995–1005
Printed in Great Britain

THE QUANTITATIVE CYTOCHEMICAL EFFECTS OF THREE METAL IONS ON A LYSOSOMAL HYDROLASE OF A HYDROID

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(Plate I, Figs. 1–3)

The quantitative effects of Cu^{2+}, Cd^{2+} and Hg^{2+} on the cytochemical staining reaction for lysosomal *N*-acetyl-*β*-D-glucosaminidase have been determined and related to the inhibitory effects of the metals on colonial growth rate in the experimentally cultured hydroid *Campanularia flexuosa*. Cytochemical threshold concentrations are comparable to known environmental levels and are about one order of magnitude lower than those obtained by measuring colony growth rates.

Pretreatment of colonies with Cu^{2+} gave no indication of tolerance adaptation, although there is evidence of the cumulative toxicity of Cu^{2+} and the possible sequestration of this metal in endodermal cell lysosomes. There is also an indication that the Cu^{2+} may exert its toxic effect by decreasing the stability of the lysosomal membranes, thus increasing the level of free glucosaminidase activity.

INTRODUCTION

It is known that vertebrate lysosomes can sequester and concentrate metal ions including copper, mercury (and organic mercurials), lead, zinc, tellurium, iron, nickel, silver and plutonium (Koenig, 1963; Rahman & Lindenbaum, 1964; Scheuer, Thorpe & Marriott, 1967; Vaughan, Bleaney & Williamson, 1967; Verity & Reith, 1967; Barrett & Dingle, 1967; Abraham, Morris & Smith 1967; Slater, 1969; Brun & Brunk, 1970). Allison (1969) states that the accumulation of certain metal ions within lysosomes may labilize the membranes under certain conditions. Furthermore, Goldfischer (1965) suggests that the toxicity of copper may not be fully manifested while it is contained within the lysosomes, but overloading of the storage capacity, or damage to the lysosomal membrane, resulting in liberation of copper into the cytoplasm could lead to cell injury. The haemocytes of *Ascidia pygmaea* have also been shown to accumulate pentavalent vanadium in a process involving sequestration by lysosomes (Kalk, 1963). It follows that quantitative cytochemical measurements of certain aspects of lysosomal function might permit the detection of responses to toxic materials at concentrations below those to which the whole organism would respond.

This work is a development of a bioassay technique, using a clonal hydroid (*Campanularia flexuosa*), which was intended primarily to study contaminants of sea water at levels comparable to those found in polluted estuaries (Stebbing, 1976). Sensitivity to low metal levels was achieved using the mean colonial growth rate over 11-day periods as an index of response. The use of a cytochemical measure of response to stress offered the likelihood of greater sensitivity, but as important to us was the possibility that

hydroids might also provide a simple system with which to study the mechanism of metal toxicity in a marine organism at the cellular level. Variability is minimized by the use of a single clone throughout this work and the cytochemistry is simplified because hydroids consist mainly of two tissues.

The usual response of hydroid colonies to natural stressors, such as lack of food or low temperatures at the onset of winter, is to degenerate (Tardent, 1963) until only a small knot of cells remains. It is from these cells that colonies are able to regenerate in the spring. Karbe (1972) has shown that this process also occurs when a stress is induced experimentally with toxic concentrations of metal ions. Degeneration is inevitably preceded by a slowing in colonial growth rate, which is the index of response we have been using in our allied study on metal toxicity (Stebbing, 1976). Degenerative processes in other organisms often involve autolysis by lysosomal hydrolases (Miller & Wolfe, 1968). One of these enzymes, namely *N*-acetyl-β-D-glucosaminidase has been used as an indicator of lysosomal hydrolase activity, and the staining reaction measured using two wavelength cytophotometry (Pollister, Swift & Rasch, 1969). Results are presented here which show the effect of three different metals on colonial growth rate relative to their effect on the cytochemical activity of *N*-acetyl-β-D-glucosaminidase.

Bryan (1974) has demonstrated the tolerance to copper of *Nereis diversicolor* from a population living in copper-rich sediments in Restronguet Creek, and we have studied the effects of pre-exposure for several weeks to low levels of copper. Such a study could possibly be indicative of lysosomal involvement in the response to metals. Physiological properties of lysosomes were investigated using modulators of lysosomal membrane permeability such as hydrocortisone and chloroquine (Reynolds, 1969; Cowey & Whitehouse, 1966). These compounds have been shown to stabilize lysosomal membranes in stressed organisms thus reducing free hydrolase activity (Weissmann, 1969).

MATERIALS AND METHODS

The hydroid, *Campanularia flexuosa*, is cultured on glass plates (1 dm^2) held in Perspex racks in static tanks (2 l). Colonies are initiated by removing uprights with three or more hydranths from stock colonies. These explants are held in position on new plates by monofilament nylon thread for 3 days before they attach themselves and the nylon can be removed. The hydroids are fed daily during experiments by immersion for 30 min in a dense suspension of newly hatched nauplii of *Artemia salina* (35–40/ml); the racks are then transferred to fresh solutions. The colony members (hydranths, gonozooids and buds) are counted at the beginning and end of each 11-day experiment and the mean growth rate (K) of each rack of seven replicate colonies calculated from the equation:

$$K = \frac{\log_e(n_y) - \log_e(n_x)}{(t_y - t_x)},$$

where t_x is the first day of an experiment and t_y the last day; n_x and n_y are the number of colony members on those days. The value of K for each treatment or group of replicates can be expressed as a percentage of K for the control group and plotted against the concentrations to which the colonies were exposed.

Experiments were all performed in sea water collected offshore near the Eddystone Rock; the water is membrane filtered (0·45 μm) before use. In each case the chlorides of the metals to be tested were used and fresh solutions were made up from stock each day, so that the nominal concentrations would be maintained.

The effect of pre-exposure to copper on the hydroids was investigated by taking colonies derived from parent stock which had been maintained in sea water containing 10 μg/l Cu^{2+} for three

weeks and comparing their response to a range of Cu^{2+} concentrations with others kept in normal sea water over the same period.

In order to examine the effects of modulators of lysosomal membrane permeability, hydroids were treated for 3 h with hydrocortisone hemisuccinate (1 mg/l) and chloroquine (10 mM) for 3 h. The effects of these compounds were tested on control colonies and colonies that had been exposed to 20 μg/l Cu^{2+} for 11 days. The modulators were added to the appropriate culture medium, i.e. filtered sea water and filtered sea water plus Cu^{2+}.

On day 11, after the colonies had been counted, all the hydroid tissue was removed from the plates and embedded in a small block of sheep heart muscle, which was used to support the hydroid tissues for cryostat sectioning. The block of muscle was then dropped into Analar hexane (aromatic hydrocarbon-free) at -70 °C for 45 s and stored sealed at -70 °C until required for sectioning. Cryostat sections (10 μm) were cut at cabinet temperature of -26 °C in a Bright's Cryostat. The haft of the knife was packed with crushed 'dry ice' and the sections were transferred to glass slides (room temperature) by bringing these close to the knife. The sections were stored in the cryostat for 30 min to 2 h before staining. Cryostat sections (10 μm) were also cut from control colonies which had been fixed overnight in Baker's formol-calcium (4 °C) and transferred to gum-sucrose at 0 °C for 24 h.

Acid phosphatase and *N*-acetyl-β-D-glucosaminidase were demonstrated in both fixed frozen and unfixed frozen sections using naphthol AS-BI phosphate (Pearse, 1968) and naphthol AS-BI glucosaminide (Pearse, 1972) as substrates with appropriate controls. For quantification, glucosaminidase was stained using a post-coupling reaction (Moore & Halton, 1976). The incubation medium contained 15 mg naphthol AS-BI glucosaminide (Sigma) dissolved in 2·5 ml 2-methoxyethanol which was made up to 50 ml with 0·1 M citrate buffer pH 4·5 containing 2·5 % (w/v) NaCl. To this medium was added 5 g of a low-viscosity collagen derived polypeptide (Sigma P5115) to act as a colloid stabilizer (Bitensky, Butcher, Chayen, 1973). Incubation time for sections in this medium was 14 min at 37 °C in a shaking water bath.

Slides were then rinsed in saline 2·5 % (w/v) NaCl for 3 min at 37 °C before being transferred to 0·1 M phosphate buffer pH 7·4 (+2·5 % NaCl) containing fast red violet LB (1 mg/ml) (Sigma), as diazonium coupler for 10 min. Sections were then fixed for 10 min in Baker's formol-calcium, rinsed in distilled water and mounted in glycerol gelatin.

Quantitative measurements were carried out using a Zeiss Microscope Photometer equipped with a Zeiss diffraction-grating monochromator. The two wave-length method of Ornstein and Patau (Pollister, Swift & Rasch, 1969) was used; the selected wavelengths (obtained from a spectral-absorption curve of a homogeneous distribution of the azo-dye) being 470 nm (λ_1) and 536 nm (λ_2). A measuring aperture of 6 μm diameter was used and section thickness was checked with the micrometer attachment on the fine focusing control. Ten areas per experimental treatment were read in the mid-cytoplasmic region of single endodermal cells in the hydroid uprights; this gave standard deviations of the mean in the range 3–10 %. Relative absorbances determined by the two wavelengths method were represented graphically as percentages of the mean control values. Measurements made on serial sections (containing all the experimental treatments) gave consistent values.

The rubeanic acid method (Pearse, 1972) was used to demonstrate Cu^{2+} in post-fixed cryostat sections of *Campanularia*. Brandino's method (Lillie, 1965) using 1 % 1,5-diphenylcarbohydrazine was used to test for Hg^{2+} in post-fixed cryostat sections.

Growth rate and cytochemical data are both presented as concentration–response curves for each metal and expressed as percentages of the mean result for the control group, so that the two responses could be presented on the same graph. From these curves arbitrary thresholds of sensitivity can be determined by relating the variability of the data to the magnitude of any departure from the 100 % level. As the metal concentration is increased and the curve rises or falls, the point at which the departure becomes significant can be calculated using the standard error (S.E.) of the control group for the cytochemical index and the pooled S.E. of all the groups of colonies for the growth rate index. Here three times the S.E. ($P = < 0{\cdot}001$) is used as the level of significance and the thresholds of sensitivity are determined from the intercepts of concentration–response curves and the 3 S.E. level above or below the 100 % line.

RESULTS

Acid phosphatase and *N*-acetyl-*β*-D-glucosaminidase were present in the ectodermal and endodermal cells of the uprights (Pl. IA–D), stolon and hydranths. The staining reaction for both enzymes was localized in small cytoplasmic granules (0·3–1·0 *μ*m) and larger spherical cytoplasmic inclusions (1–8 *μ*m) (Pl. IA, B). These cytochemical distributions remained the same in sections which were frozen in hexane (−70 °C) and

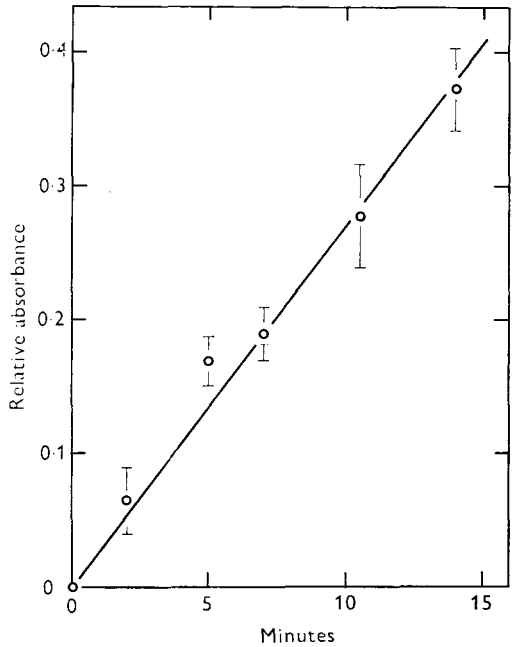

Fig. 1. Cytochemical reaction curve for *N*-acetyl-*β*-D-glucosaminidase determined cytophotometrically in endodermal cells.

cryostat sections of hydroids fixed in Baker's formol-calcium. Pre-treatment of hexane-frozen sections for 1 min with 0·2% Triton X-100 in 3% NaCl + 5% polypeptide markedly reduced the level of staining for these hydrolytic enzymes. Treatment of fixed sections with this detergent produced a less drastic decrease in the staining of the small cytoplasmic granules for glucosaminidase. The granule and vacuole associated distribution of staining within the endodermal cells indicates that both of the hydrolytic enzymes were probably lysosomal. The staining reactions also demonstrated that the larger cytoplasmic inclusions form part of the lysosomal vacuolar digestive system.

Cytophotometric measurements showed that the hydrolytic release of naphthol AS-BI is linear over the incubation period used for the comparative study of control and experimentally treated groups (Fig. 1).

The results of representative experiments with cupric chloride, cadmium chloride and mercuric chloride are presented as a series of concentration–response curves (Fig. 2). At subthreshold metal concentrations, growth occurs at a rate similar to that of the control

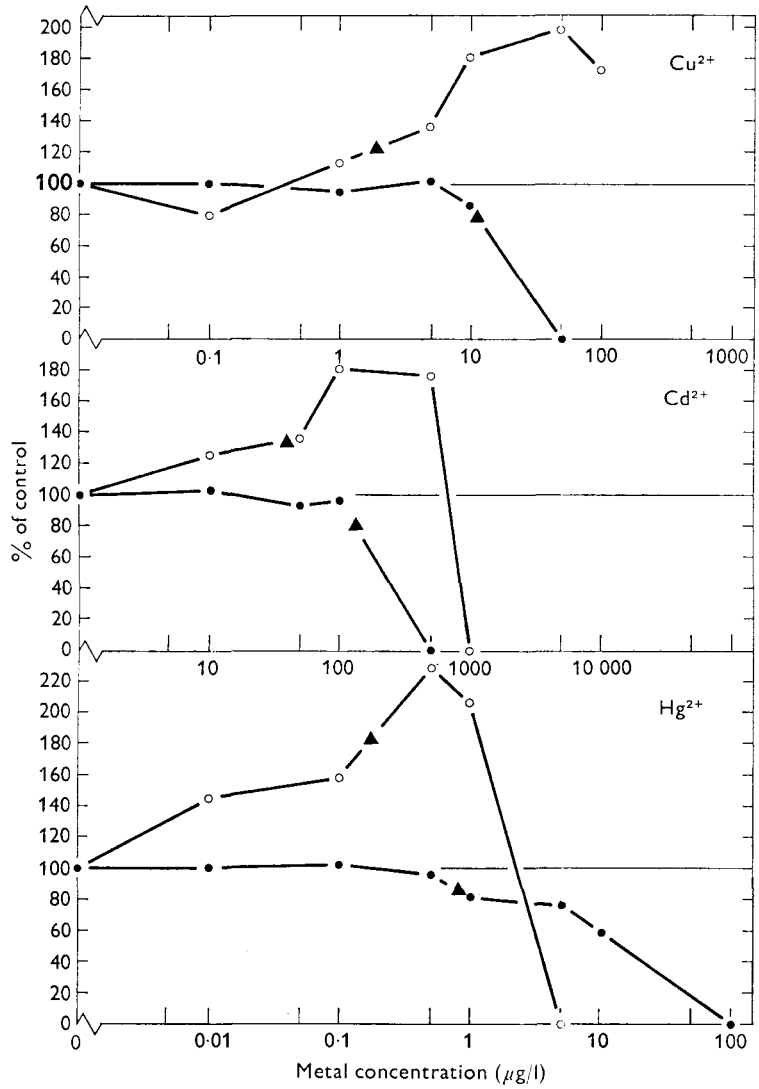

Fig. 2. Concentration–response curves for the effects of Cu^{+}, Cd^{2+} and Hg^{2+} on *Campanularia flexuosa* colonies. Both the glucosaminidase reactivity (○) and the growth rate data (●) are expressed as percentages of the response of the control group of colonies. The method for determining the thresholds of sensitivity (▲) is described in the text.

group but at higher concentrations the metal takes effect and the growth rate is inhibited, the curve falling sharply from the control level. Curves showing changes in staining intensity (relative absorbance) of *N*-acetyl-*β*-D-glucosaminidase staining reaction in the same colonies follow a different, although equally consistent pattern. Initially there is a gradual increase in the enzyme staining reaction (Pl. Ic, D), which may reach a level 50–150% greater than that of the control colonies. After reaching a peak, enzyme activity as represented by the intensity of the staining reaction, in each case falls rapidly with further increase in metal levels.

The cytochemical (lysosomal enzyme) index of the hydroid's response to metals is more sensitive than colonial growth rate; increased enzyme staining reaction is detectable at metal concentrations one order of magnitude lower than those at which growth rate becomes inhibited significantly (Table 1). Reproducibility is best seen in the experiment with cupric chloride, which has now been repeated four times. The threshold concentration for the inhibition of growth rate in the first experiment is shown in parentheses because a different subculturing technique resulted in erratic early growth and an artificially low

TABLE 1. GROWTH-RATE AND CYTOCHEMICAL THRESHOLD VALUES FOR EXPOSURE TO COPPER, CADMIUM AND MERCURY

Metal	Exp. no.	Threshold for colonial growth (μg/l)	Threshold for cytochemical index (μg/l)
Cu^{2+}	1	(2·4)*	1·20
	2	13	1·39
	3	10	1·90
	4	12	1·24
	5	18	—
Cd^{2+}	1	110	40
	2	280	75
Hg^{2+}	1	1·6	—
	2	1·7	0·17

* A different subculturing technique was used in this experiment.

threshold concentration. It was to be expected that the cytochemical lysosomal response would be unaffected as this is a specific response to the metal ions, while the growth rate can be inhibited by other factors. The effect of cadmium chloride on growth rate is such that the concentration–response curve falls very steeply. Consequently, the choice of concentrations and the intervals between them affect the shape of the curve, and the threshold concentrations derived from it. The differences between the threshold concentrations for both parameters reflect the importance of this. The second experiment gives the more reliable result, because there is an extra concentration in the range where effects are occurring.

An experiment was designed to investigate possible changes induced by prior exposure to low copper levels. Colonies were maintained under the normal experimental regime for 3 weeks in 10 μg/l Cu^{2+}. Sub-cultures were then made from these pre-exposed colonies for comparison with a duplicate set subcultured from normal parent stock. The two sets of colonies were then exposed to three copper concentrations in an experiment lasting 11 days. The results show no evidence of tolerance among the pre-exposed colonies; on the contrary, those colonies at 25 μg/l Cu^{2+} proved more sensitive to subsequent exposure (Fig. 3). It is not surprising that only the intermediate copper level showed the effect of pretreatment on growth rate, because the lowest concentration had no effect on growth rates and the effect of the highest level was total. Only between these extremities of the range of response was there scope for pre-exposure to have an effect. This was not the case with the cytochemical index, which we know to be responsive to both higher and lower copper levels. The effect of pretreatment is therefore apparent over the whole range of concentrations as consistently more intense staining

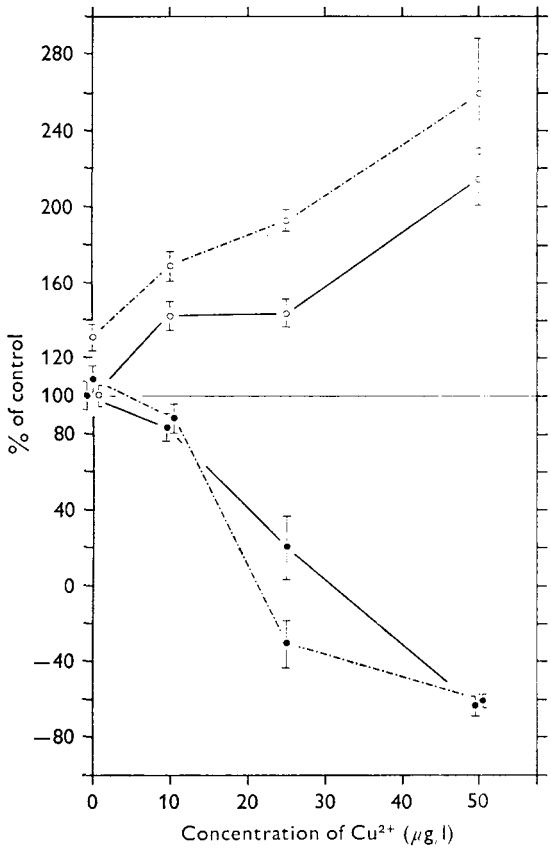

Fig. 3. The effects of Cu^{2+} on the growth rate (●) and glucosaminidase reactivity (○) of *Campanularia flexuosa* colonies which have been pre-exposed to 10 μg/l Cu^{2+} for 3 weeks (– – –) compared with those that have not been pre-exposed (——). The vertical bars mark the standard error for each group of seven replicate colonies and all results are expressed as percentages of the response of the control group of colonies subcultured from normal parent stock.

TABLE 2. THE EFFECTS OF HYDROCORTISONE AND CHLOROQUINE ON LYSOSOMAL GLUCOSAMINIDASE STAINING INTENSITY (RELATIVE ABSORBANCE)

Treatment	Relative absorbance (% of control)	Significance	(t test)
Controls	100	NS*	NS
+Hydrocortisone	87·7		
+Chloroquine	93·4		
Cu^{2+} (20 μg/l) treated	155·5	$P < 0.05$	NS
+Hydrocortisone	115·2		
+Chloroquine	130·1		

* NS = not significant.

reactions for glucosaminidase ($P < 0{\cdot}025$) in the pre-exposed hydroids. The results suggest that the response of the hydroids is probably related to the internal metal levels and the pre-exposed colonies were more sensitive because they had already accumulated some copper and therefore reached internal threshold levels sooner than the others.

It is interesting to note that the pre-exposed colonies which had been returned to sea water gave a staining reaction 50% greater than the control colonies, indicating that the effect of Cu^{2+} was not reversible over a period of 11 days.

Hydroids exposed to 20 μg/l of Cu^{2+} for 11 days and treated with hydrocortisone hemisuccinate (1 mg/l) for 3 h showed a distinct difference from hydroids which were not incubated with the hormone (Table 2). The effect of the hormone was to reduce the staining reaction of the Cu^{2+} treated hydroids from 155·5% to 115·2% ($P = < 0{\cdot}05$). Chloroquine (10 mM) did not induce a significant alteration in the intensity of the staining reaction. Hydrocortisone and chloroquine have no significant effects on the endodermal cells of control hydroids not treated with Cu^{2+} (Table 2). In post-fixed sections of Cu^{2+} treated hydroids stained with rubeanic acid, black-green coloration indicative of copper was observed in the non-cellular perisarc at a concentration of 1 μg/l. Staining was also associated with small cytoplasmic granules and larger inclusions with a similar distribution to the cellular components identified as lysosomes in hydroids incubated at 50 μg/l and higher. No staining for Hg^{2+} could be detected in hydroids using Brandino's method.

DISCUSSION

The metal-induced degenerative process (Stebbing, 1976) in hydroid colonies involves increased lysosomal hydrolase activity as reflected by *N*-acetyl-β-D-glucosaminidase. Reactivity for this enzyme, as well as acid phosphatase, was localized in large vacuoles and small cytoplasmic granules in the endodermal cells of *Campanularia*, indicating a lysosomal distribution. This evidence is reinforced by the observed effects of Triton X-100 which labilizes lysosomal membranes (Desai, 1969; Tiffon, 1971), and by the morphological studies of Lunger (1963) on *Campanularia*. The results show that this cytochemical technique for glucosaminidase demonstrates a significant response to extremely low levels of toxic metal ions. The form of the response curve is similar for all three metal ions, with a gradual increase in glucosaminidase staining reaction with increasing metal concentration. The peak corresponds to the concentration at which growth rate slows, indicating that a major physiological change occurs at this concentration.

The toxic effects of Cu^{2+} were shown to be cumulative by comparing the concentration–response curves for hydroids which previously had been exposed in sea water containing 10 μg/l of $CuCl_2$ for 3 weeks with those not pre-exposed to copper. Data from both the growth analysis and the cytochemistry confirmed this evidence. It is also interesting to note that the Cu^{2+} toxicity persisted for nearly two weeks, in clean sea water.

The mechanism of copper toxicity, and possibly that of Hg^{2+} and Cd^{2+} may be related to decreased stability of the lysosomal membranes. This would result in higher levels of free glucosaminidase activity, rather than an increase in either the number of lysosomes or the actual amount of lysosomal hydrolase.

This hypothesis is supported by results from the treatment of hydroids subject to Cu^{2+} (20 μg/l), with hydrocortisone which is known to stabilize lysosomal membranes (Reynolds, 1969). The hydrocortisone effectively reduced the staining reaction for glucosaminidase to the control level. An analogous situation has recently been demonstrated in cultured peritoneal macrophages exposed to quartz powder (Kaw, Beck & Bruch, 1975), where lysosmoal membrane stability was restored by treatment with polyvinylpyridine-*N*-oxide.

In the experiments with $CuCl_2$, sections stained with rubeanic (dithio-oxamide) acid showed that accumulation of Cu^{2+} occurs in cytoplasmic structures believed to be lysosomes. Deposits of copper ions were stained in hydroid endodermal cells exposed to a concentration of 50 μg/l, showing that the endodermal cells were capable of sequestering and accumulating this metal. It has not proved possible yet to demonstrate accumulations of Cd^{2+} and Hg^{2+} at similar sites, but the similarity of the response curves for these ions and that for Cu^{2+} suggests that a similar mechanism may be involved.

The results obtained with the hydrocortisone indicate that the toxicity of copper may be mediated by a reduction in the stability of the lysosomal membranes, thus increasing the level of free glucosaminidase activity. However, this decrease in stability may also be accompanied by autophagy (Ericsson, 1969; Moore, 1971, Ph.D. Thesis, Queen's University of Belfast; Threadgold & Arme, 1974; Moore & Halton, 1973, 1976) particularly at higher concentrations of metal ions. The peaks of enzyme activity correspond to the threshold concentrations obtained from growth analysis, suggesting that cellular degeneration in *Campanularia* may be related to increased lysosomal glucosaminidase activity. In this instance, autophagy could be active either as a physiological survival mechanism or as a pathological response, or perhaps a combination of both (Ericsson, 1969).

A comparison between the sensitivity of the hydroid bioassay technique and others used in marine toxicological studies has already been made by Karbe (1972) and Stebbing (1976) who has demonstrated that the growth rate analysis technique is sensitive to metal ion concentrations of the same order as those sometimes found in sea water. The cytochemical index of glucosaminidase activity clearly improves this sensitivity by an order of magnitude, without loss of reproducibility. Further experimental work should extend the applications of this cytochemical bioassay technique.

The authors wish to thank Mr D. Nicolson (M.B.A.) and Mr N. Cotter for the preparation of figures and photomicrography, and Mr D. Lowe, Mr A. Pomroy, Mrs P. Feith and Mrs S. Moore for technical assistance. The work forms part of the experimental ecology programme of the Institute for Marine Environmental Research, a component of the Natural Environment Research Council. It was commissioned in part by the Department of the Environment. (Contract No. DGR 480/47.)

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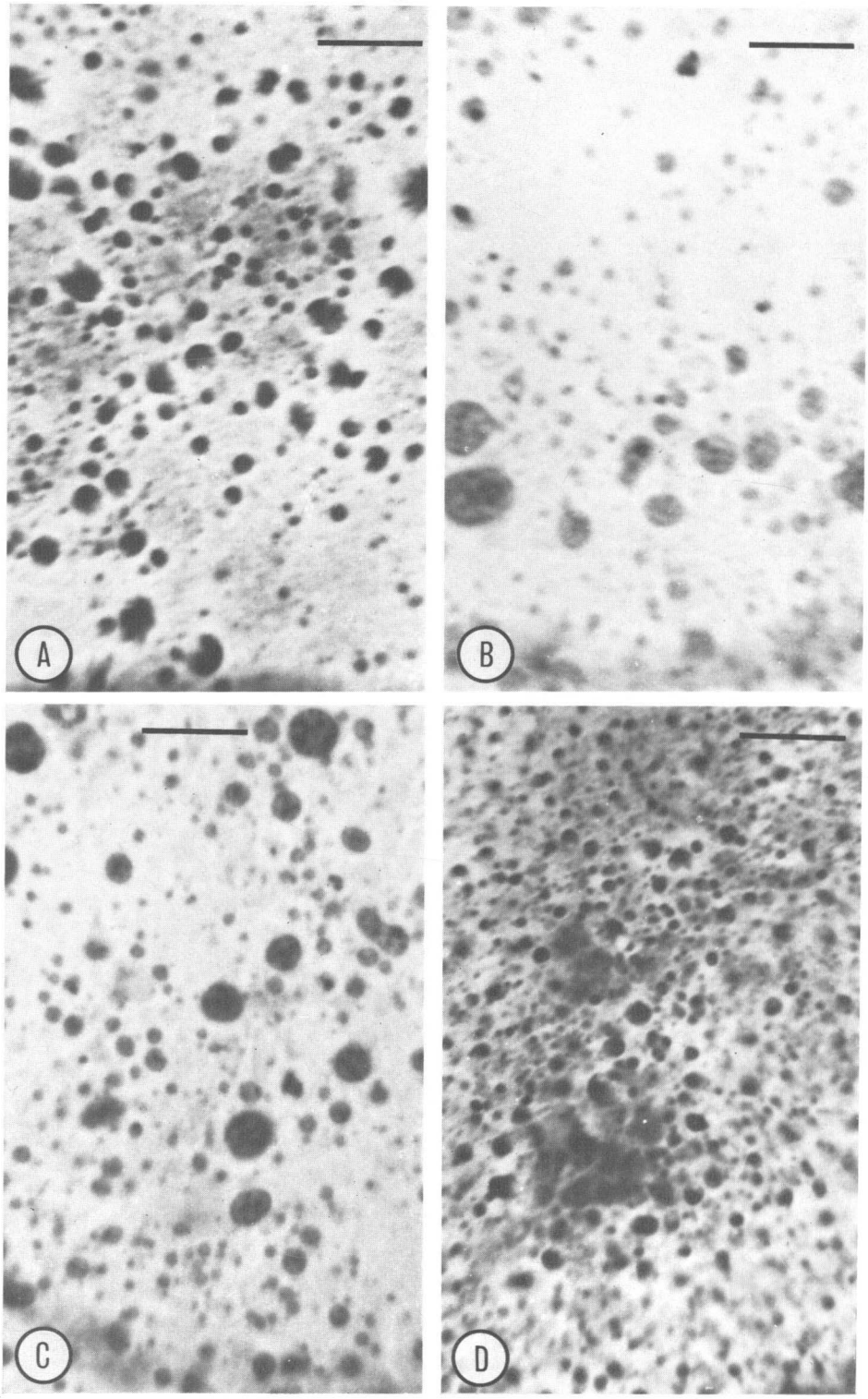

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EXPLANATION OF PLATE

Scale Bar = 10 μm.

(A) A fresh frozen section through endodermal cells of the upright showing the localization of acid phosphatase activity in lysosomes. Naphthol AS-BI phosphate method. × 1475, oil.

(B) Section as in (A) of endodermal cells in a control animal showing *N*-acetyl-β-D-glycosaminidase activity in lysosomes. Naphthol AS-BI glucosaminide method. × 1475, oil.

(C) Section as in (A) of endodermal cells in an experimental animal treated with 20 μg/l of Cu^{2+} for 11 days showing an increased staining reaction for glucosaminidase in the lysosomes. Naphthol AS-BI glucosaminide method. × 1475, oil.

(D) Section as in (A) of endodermal cells in an experimental animal treated with 0·5 μg/l of Hg^{2+} for 11 days showing an increase in the staining reaction for glucosaminidase in the lysosomes. There is also an increase in the number of lysosomes. Naphthol AS-BI glucosaminide method. × 1475, oil.