Lysosomal membrane fragility and catabolism of cytosolic proteins: evidence for a direct relationship

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Summary. A direct relationship was established between the stability of the lysosomal membrane and an estimate of cytosolic protein catabolism, based on loss of radiolabel from prelabeled protein. Lysosomes in the lysosomally-rich digestive cells of the midgut gland of the marine mussel *(Mytilus edulis)* were destabilized by experimental treatment with phenanthrene.
Key words. Lysosomes; lysosomal stability; protein catabolism; digestive cells; marine mussel; phenanthrene.

Lysosomes are known to be involved in the catabolism of intracellular proteins; the precise mechanisms for the uptake of these proteins are not clearly identified although there are indications that both macroautophagy and microautophagy may be involved[1]. The stability of the lysosomal membrane can be reduced by a wide range of endogenous and environmental stimuli, leading to reduced hydrolase latency as a result of increased permeability and fluidity of the membrane[2,3]. The consequences of this type of membrane alteration for lysosomal protein catabolism are not altogether clear, although there are indications of increases in the rates of protein breakdown and vacuolar fusion[4-6]. In view of the importance of the lysosomal system in turnover of cytoplasmic components, and its involvement in many pathological responses[5], it is essential to elucidate the effects of lysosomal membrane labilizing agents on in vivo lysosome function.

In the present investigation, the experimental animal is the marine mussel *(Mytilus edulis)* as the molluscan midgut or digestive gland (hepatopancreas) is probably one of the richest source of lysosomes in the animal kingdom. The midgut gland is composed of myriad blind ending tubules into which food is passed for intracellular digestion. The food material is endocytosed into the epithelial cells lining these tubules, largely composed of columnar epithelial cells termed digestive cells. These cells are extremely rich in lysosomes which can frequently be 5 µm or more in diameter[7,8]. The digestive cell lysosomes are involved in the breakdown of intracellular proteins[4], and can be destabilized by a variety of agents including 17β-estradiol, progesterone and xenobiotics such as the polycyclic aromatic hydrocarbon phenanthrene[9-12]. In this study we have investigated the effects of phenanthrene on cytochemically determined lysosomal membrane fragility and the rate of protein catabolism based on an evaluation of the loss of ^{14}C-radiolabel from prelabeled cytosolic proteins in the midgut gland. Phenanthrene was chosen because being a lipophilic compound it is likely to directly alter the membrane structure mainly by penetrating the lipid bilayers[13]; the involvement of reactive metabolites of phenanthrene is probably relatively minimal due to the low activities of cytochrome P-450 monooxygenase activity in the digestive cells[14].

Lysosomal membrane fragility was determined utilizing a cytochemical procedure as even gentle homogenization results in extensive damage to the larger lysosomes in the lysosomal fraction (Moore and Bayne, unpublished data)[4].

Materials and methods. Mussels were collected from the Exe Estuary (Devon) and maintained in the laboratory at 15 °C with an adequate supply of mixed algal food (> 30 mg dry wt algal material assimilated/mussel/day) for 10 days. All mussels measured between 45–55 mm shell length. Experimental exposures to phenanthrene were carried out in seasoned polypropylene containers each holding 7 liters of filtered (0.8 µm) seawater with 7 mussels per treatment at 15 °C. Phenanthrene (Aldrich) was dissolved in Analar acetone (10 mg·ml^{-1}) and rapidly injected into the seawater which was continuously mixed. Mussels were not fed during exposure experiments. Control conditions received acetone (40 µl·l^{-1}) and acetone concentrations were standardized throughout the treatments.

Experimental design. Mussels were exposed to a concentration range of phenanthrene (0, 25, 50, 100, 125, 150, 200 and 400 µg·l^{-1}) for 24 h in order to determine a dose response relationship for lysosomal membrane fragility[15,16]. Five mussels were sampled for each treatment.

For the determination of the response period of lysosomal membrane fragility mussels were exposed to 200 µg phenanthrene l^{-1} for 0, 3, 6, 12 and 24 h.

Experiments to determine the effects of phenanthrene on the loss of ^{14}C-label from midgut gland cytosolic protein and lysosomal membrane fragility of digestive cells used phenanthrene concentrations of 0, 50, 100, 150 and 200 µg l^{-1} for 24 h. Each sample consisted of 5 animals and all treatments were replicated between 5 and 11 times. Exact number of replicate treatments were

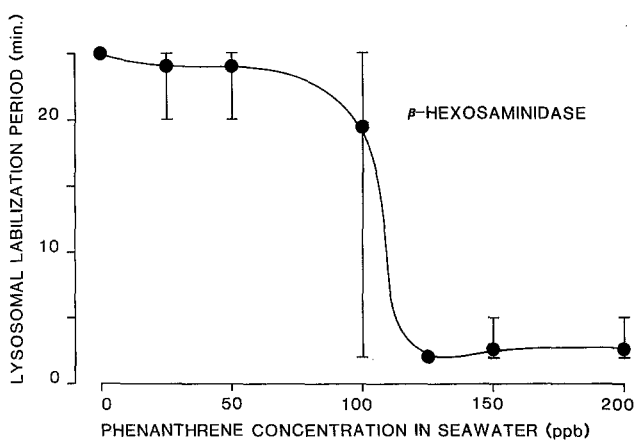

Figure 1. The effect of phenanthrene concentration in seawater on lysosomal stability in digestive cells, based on labilization period of latent β-N-acetylhexosaminidase (n = 5, point indicates mean value, bars indicate data range except where these are too small to show on the figure).

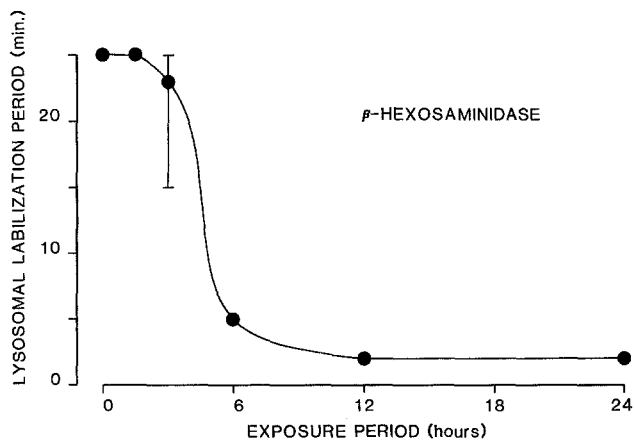

Figure 2. The effect of exposure to an initial concentration of 200 µg phenanthrene·liter^{-1} on lysosomal stability in digestive cells, over a period of 24 h (n = 5, point indicates mean value, bars indicate data range except where these are too small to show on the figure).

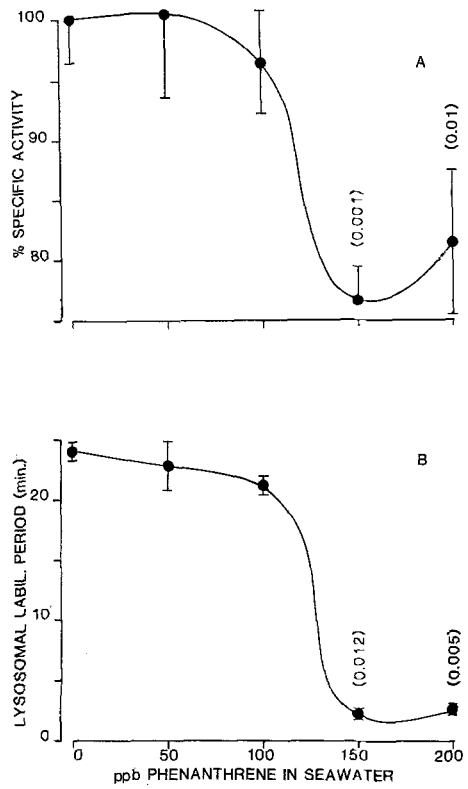

Figure 3. *A* The effect of phenanthrene concentration in seawater on specific activity of ^{14}C-labeled cytosolic proteins (as % of control values) in the midgut gland (each point is the mean ± SE of a least 5 replicate experiments, as indicated in the methods section). *B* The effect of phenanthrene concentration in seawater on lysosomal stability in digestive cells (each point is the mean ± SE of 5 replicate experiments, each experimental treatment contained 5 animals). Exact probability value are given in the figures, where values differ significantly from those of the control. These are shown vertically above the appropriate data points.

as follows: control, 11; 50 µg l^{-1}, 7; 100 µg l^{-1}, 5; 150 µg l^{-1}, 5; 200 µg l^{-1}, 6.

Cytochemical procedure for lysosomal membrane fragility (labilization period). This involved a slight modification of the method described by Moore[16]. Small pieces (approximately 3–5 mm^3) of freshly excised midgut gland were supercooled in n-hexane at −70 °C. By following this procedure there is no formation of ice and hence no structural damage to the subcellular components[15]. Cryostat sections (10 µm) were prepared in a Bright's Cryostat (motorized cutting) with the cabinet temperature below −25 °C and with the knife cooled with crushed solid CO_2. The sections were transferred to 'warm' slides (i.e. 20 °C or room temperature) which effectively flash-dried them and the slides stored in the cryostat cabinet until required[15].

Serial cryostat sections prepared as described above were pretreated in a staining jar containing 0.1 M citrate buffer (pH 4.5) containing 2.5% NaCl (w:v) at 37 °C in order to labilize the lysosomes thus freeing the latent or bound enzyme[15,16]. The pretreatment sequence commenced at 25-min down to 0-min intervals (i.e. 25, 20, 15, 10, 5, 2, and 0 min). The zero pretreatment represents the free lysosomal activity. Following this pretreatment sequence, the slides were transferred to the substrate incubation medium containing 20 mg naphthol AS-BI-N-acetyl-β-D-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% NaCl (w:v) and 3.5 g of low viscosity polypeptide (Sigma POLYPEP P5115) to act as a section stabilizer[15,16]. Incubation time was 20 min at 37 °C in a staining jar in a shaking water-bath. The slides were subsequently rinsed in 3.0% NaCl at 37 °C for 2 min before being transferred to 0.1 M phosphate buffer pH 7.4 containing the diazonium coupler fast violet B (Sigma; 1 mg · ml^{-1}) at room temperature for 10 min. The slides were then rinsed rapidly in running tap water, fixed for 10 min in calcium formol containing 2.5% NaCl (w:v) at 4 °C, rinsed in distilled water and mounted in aqueous mounting medium (Difco UV-free aqueous mounting medium).

The labilization period is the period of pretreatment required to labilize the lysosomal membranes resulting in maximal reaction product for β-N-acetylhexosaminidase. This can be accurately assessed by microscopical determination of maximum reactivity (reaction intensity) for lysosomal β-N-acetylhexosaminidase in the series of sections (i.e. pretreated for 0, 2, 5, 10, 15, 20 and 25 min). The increased reactivity represents the activation of the latent lysosomal enzyme, however, there is frequently only a partial freeing of the latent activity probably indicating that some lysosomes in the digestive cells are more susceptible to effectors than others; this is visualized cytochemically as two separate peaks of enzymic reaction product[11,12]. Hence, the labilization period used in this investigation is the pretreatment time required to give either a single peak of maximal reactivity or the first peak if two occur.

Selected microscopical determinations of labilization period were subsequently checked using microdensitometry (Vickers M85) with a measuring area of 38 $µm^2$ and a wavelength of 560 nm[12].

Evaluation of the rate of protein catabolism. The procedure adopted represents a modification of the decay curve method[17]. Groups of 8–9 mussels were exposed to ^{14}C-leucine (specific activity = 300 mCi · m $mole^{-1}$) for 24 h (1.5 µCi $animal^{-1}$). Subsequently the animals were maintained for 12 h in seawater containing a high concentration of unlabeled leucine (200 mg · l^{-1}; 1 animal per liter). The mussels were then exposed to phenanthrene for 24 h to evaluate the effect of the organic xenobiotic on the loss of radioactivity from the gland cytosolic proteins. Mussels were exposed to unlabeled leucine (50 mg · l^{-1}; 1 animal per liter) during the period of treatment.

Evaluation of the specific activity of the cytosolic proteins. The midgut gland was removed from the mussels, washed with a solution of 0.5 M sucrose containing 0.15 M NaCl buffered with 20 mM Tris-HCl, pH 8.6 and homogenized in 2 vol (w:v) of the same solution. The homogenate was centrifuged at 30,000 × g for 20 min at 0–4 °C. The supernatant was then centrifuged at 100,000 × g for 90 min at 0–4 °C to obtain the cytosolic fraction. Aliquots of 25 µl of the cytosols were used to analyze protein content. Aliquots of 100 µl of cytosol were spotted onto Whatman 3 MM filter paper squares (2.3-cm edge length) and utilized to evaluate the incorporation of the labeled precursor into proteins, essentially as described by Yu and Feigelson[18]. The squares were then extracted 4 times in 5% TCA at 0 °C to remove the acid soluble radioactivity. Filters were successively extracted for 20 min at 90 °C, and then further washed twice with cold 5% TCA, twice with ethanol-ether mixture 1:1 (v/v) and with ether before being dried. Radioactivity values, due to the incorporation of the labeled amino acid into the protein fraction, were expressed per mg of cytosolic proteins.

Protein assay. Proteins from the cytosol were assayed according to the Harthree method using serum albumin fraction V as a standard[19].

Measurement of radioactivity. Radioactivity was measured in a Packard 2425 liquid scintillation spectrometer. Each sample was counted in 5 ml of Instagel for enough time to assure a counting error no greater than 5%.

Statistical analyses. All experimental treatments were compared to the control conditions using the non-parametric Mann-Whitney U-test[20,21].

Results. Effects of phenanthrene concentration on lysosomal membrane stability. The lysosomes of hepatopancreatic digestive cells

of mussels exposed to phenanthrene in seawater at concentrations of 0–400 $\mu g \cdot l^{-1}$ (1 mussel per liter seawater) for 24 h showed a dose-dependent response as illustrated in figure 1. This response pattern has been confirmed in five separate experiments. Figure 1 shows that there is a critical concentration at which destabilization of the lysosomal membrane occurs and this response is apparent at 125 $\mu g \cdot l^{-1}$ of phenanthrene ($p < 0.01$). At concentrations of phenanthrene greater than 125 $\mu g \cdot l^{-1}$ there was no further effect on the lysosomes.

Response period of lysosomal membrane stability following exposure to phenanthrene. The relationship between time of exposure to phenanthrene (200 $\mu g \cdot l^{-1}$) and lysosomal membrane stability is illustrated in figure 2. Phenanthrene had no effect until 6 h after exposure ($p < 0.01$) indicating that a critical concentration of phenanthrene within the lysosomes had been reached as indicated by the dose response relationship (fig. 1). These data also show an all or nothing type of response.

Effects of phenanthrene on loss of ^{14}C-labeled cytosolic protein. Due to the complexity of interpretation of the in vivo results concerning the rate of protein catabolism it is important to mention the main features of the procedure used. The method employed requires the treatment of the ^{14}C-leucine exposed mussels with a high concentration of unlabeled leucine. This pulse chase procedure decreases the specific activity of the soluble cytosolic leucine present in the digestive gland cells, to values ranging from 11% to 6% of those found in the mussels that received the same treatment with the radioactive precursor but not exposed to unlabeled leucine: in this way the reincorporation in the neosynthesized proteins of radiolabeled leucine coming from protein catabolism is minimized[21].

In addition the concentration of cold leucine utilized does not significantly alter the stability of the lysosomal membrane, nor the total amount of free amino acids in the digestive gland cells, the levels of leucine present in the soluble amino acid pool changing from about 0.3%[23] to 4–5% during the 36-h experiment[22]. The effect of a concentration range of phenanthrene on the specific activity of ^{14}C-labeled cytosolic proteins are illustrated in figure 3A. These results showed that phenanthrene significantly decreased the concentration of ^{14}C-labeled cytosolic protein after 24 h at 150 and 200 μg phenanthrene $\cdot l^{-1}$ (fig. 3A); lysosomal stability was also significantly reduced at these concentrations (fig. 3B). Both lysosomal membrane stability and percentage concentration of ^{14}C-labeled cytosolic proteins were significantly correlated ($r = 0.994$, $n = 5$, $p < 0.001$, two-tailed test).

Discussion. The results show that phenanthrene induces an all or nothing type of effect on the fragility of the lysosomal membrane in the midgut gland digestive cells. This suggests that at a certain level of incorporation of phenanthrene into the lysosomes, and presumably into the lipid bilayer of the membrane, there is a 'catastrophic' alteration in membrane organization involving permeability/fluidity[24] resulting in the reduced stability of latent β-N-acetylhexosaminidase as measured cytochemically. Effects of this type have been observed in digestive cell lysosomes exposed to 17 β-estradiol[11], in lysosomes from rat preputial gland exposed to 17 β-estradiol[2,3], and in the thyroid follicular cells exposed to thyroid stimulation[25,26]. The response period data lends support to the hypothesis that a critical concentration of phenanthrene must be accumulated by the lysosomes before there is a destabilizing effect of the membranes. A response period of 3–6 h (fig. 2) is longer than that observed with 17 β-estradiol[11], however, the very low dose of the steroid (2.8 ng 17β-estradiol $\cdot$ g mussel tissue^{-1}) required to induce a response suggests the mediation of a receptor system[2,3], which would not be the case with phenanthrene.

The decrease in specific activity of ^{14}C-labeled cytosolic proteins induced by phenanthrene indicates that there is an increased loss of ^{14}C-label from the animals, presumably due to a stimulation of the net degradation of protein. As the possibility of reincorporation of ^{14}C-label due to protein synthesis has been minimized, this finding is consistent with enhanced catabolism of cytosolic proteins. Furthermore, as most protein catabolism is believed to occur in the lysosomal system, the concomitant reduction in lysosomal membrane stability is probably related to the increased capacity of the lysosomes for protein catabolism. Destabilization of the lysosomes, involving as it does increased permeability and fluidity of the lysosomal membrane probably facilitates fusion with other membrane bound vesicles and hence entry of cytoplasmic materials into the lysosomal system[5].

The results presented above support the hypothesis that increased fragility of lysosomal membranes is directly related to enhanced protein catabolism. Further support is derived from the evidence of elevated concentrations of intralysosomal free amino acids following lysosomal destabilization in mussels[4], as well as ultrastructural evidence of increased autophagy and vacuolar fusion[6].

In conclusion, lysosomal membrane stability appears to be an important factor in the regulation of intracellular protein catabolism and the mechanism presumably involves molecular rearrangements within the lysosomal membrane. Further tests of this hypothesis are suggested by the findings such as the use of lysosomal membrane stabilizing agents (eg. hydrocortisone and dexamethasone) to examine the effects of increased membrane stability on cytosolic protein breakdown.

Acknowledgment. This work is part of the cellular processes research program of the Institute for Marine Environmental Research, a component of the Natural Environment Research Council. It was supported in part by a grant to A. Viarengo from the British Council to whom we are indebted.

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0014-4754/87/0320-04$1.50 + 0.20/0
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Insulin-like effect of dichloroacetic acid on hexose transport in Swiss 3T3 cells[1]

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Summary. Hexose transport in Swiss 3T3 cells was increased by treatment with dichloroacetic acid as well as by treatment with insulin. Neither extra- nor intracellular Ca^{2+} was found to be involved in their stimulatory action. On the other hand, the removal of intracellular Mg^{2+} resulted in a loss of the stimulation. These results suggest that dichloroacetic acid stimulates the hexose transport in Mg^{2+}-dependent manner, similar to that of insulin.
Key words. Hexose transport; dichloroacetic acid; insulin; divalent cation; mouse fibroblast.

Dichloroacetic acid is known as a specific activator of pyruvate dehydrogenase which inhibits the transformation of the enzyme into the inactive phosphorylated enzyme[2], and as an inhibitor of the phosphorylation of several mitochondrial proteins[3]. Insulin has also been shown to stimulate pyruvate dehydrogenase activity by activating pyruvate dehydrogenase phosphatase[4,5]. From this point of view, these agents appear to show a similar effect on this enzyme.

The stimulation of hexose transport is one of the main actions of insulin, and the analysis of the mechanism of this phenomenon has been carried out extensively. Recently, it has been reported that insulin stimulates hexose transport by translocation of the carrier from intracellular membranes to plasma membranes[6,7]. Moreover, Mg^{2+}, rather than Ca^{2+} was found to be essential for the stimulatory action of insulin on hexose transport in muscle[8], adipocytes[9] and cardiocytes[10].

In the work described in this paper we examined the effect of dichloroacetic acid on hexose transport in Swiss 3T3 cells and compared it to the effect of insulin. The results show that dichloroacetic acid has an insulin-like stimulatory effect on the hexose transport and that Mg^{2+} may be involved in the transmission of the signal of this compound which induces hexose transport in Swiss 3T3 cells.

Materials and methods. Cell culture. Swiss 3T3 cells were prepared by plating 3×10^5 cells/dish (35 mm in diameter) in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. Cells were grown in a plastic tissue plate in a humidified CO_2 incubator at 37 °C. After 3 days, cultures were used for experiments.

Measurement of hexose uptake. The uptake of 3-O-methylglucose by Swiss 3T3 cells was measured as described previously[11]. Cells were rinsed with 2 ml of phosphate-buffered saline. The uptake was initiated by addition of 1 ml of phosphate-buffered saline containing 4 μM 3-O-methylglucose (0.1 μCi/ml). After the cells had been incubated for 2 min at room temperature, uptake was stopped by washing the cells with ice-cold phosphate-buffered saline. The attached cells were dissolved in 1 ml of 0.1 N NaOH/0.1% sodium dodecyl sulfate solution, and aliquots of the lysate were taken for assay of radioactivity and determination of protein concentrations[12].

Table 1. Effect of dichloroacetic acid and its derivatives on hexose transport

Addition	3-O-Methylglucose uptake (pmol/mg protein/min)
None (control)	2.70 ± 0.12
Monochloroacetic acid (250 μM)	3.06 ± 0.47
Dichloroacetic acid (250 μM)	6.09 ± 0.60*
Trichloroacetic acid (250 μM)	3.81 ± 0.18
Dichloroacetamide (250 μM)	4.40 ± 0.38

Cells were washed and the medium was replaced with Dulbecco's modified Eagle medium containing each agent. After 2 h, the cultures were used for uptake assay as described in 'Materials and methods'. The data are expressed as mean ± SE (n=3). *$p < 0.01$ (vs control value).

Materials. [^{3}H]-3-O-Methylglucose (5 Ci/mmol) and [^{3}H]-L-glucose (10.7 Ci/mmol) were purchased from New England Nuclear. A23187 was obtained from Calbiochem and insulin was from Sigma Chemical Co. Inc. All other chemicals were obtained from commercial sources and were either of reagent grade or the highest purity available.

Results. Dichloroacetic acid stimulated the hexose transport activity of Swiss 3T3 cells (table 1). The stimulatory effect of dichloroacetic acid was time- and dose-dependent, and showed half-maxima at 40 min and 100 μM, respectively. The effect of dichloroacetic acid was completely reversed by washing of the cells (data not shown). Mono- or trichloroacetic acid at 250 μM concentration did not stimulate hexose transport significantly. Dichloroacetamide stimulated hexose transport less effectively than dichloroacetic acid at the same concentration.

Table 2 shows the effect of extra- and intracellular divalent cations on basal, dichloroacetic acid- and insulin-stimulated hexose transport. Removal of extracellular Ca^{2+} and Mg^{2+} by treatment of cells with ethylenediaminetetraacetic acid (EDTA) had no effect on the basal transport or on the transport stimulated by dichloroacetic acid or insulin. When EDTA and A23187 were added to the culture medium to remove both intra- and extracellular Ca^{2+} and Mg^{2+}, the stimulatory effect of dichloroacetic acid or insulin on hexose transport was completely suppressed. In order to exclude the possibility that the inhibition of dichloroacetic acid- or insulin-induced stimulation of hexose transport by EDTA plus A23187 may be caused by their general cytotoxicity, the effect of the restoration of Ca^{2+} or Mg^{2+} was examined. The inhibitory effect of these agents was removed by restoration of Mg^{2+} to the culture medium, On the other hand, it was difficult to draw conclusions as to whether the restoration of Ca^{2+} was effective in reversing the inhibition, since the basal uptake of 3-O-methylglucose was elevated by increased cytosolic Ca^{2+}.

Discussion. The present study shows that dichloroacetic acid stimulates hexose transport, and that this depends upon Mg^{2+} in similar manner to stimulation by insulin. Mg^{2+} appears to play an important role in the regulation of hexose transport in various cells. In muscle, hexose transport is regulated by an intracellular magnesium pump and stimulated through an increase in