LYSOSOMES AND L-ASCORBIC ACID

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

THE UNIVERSITY OF MANITOBA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

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A thesis submitted to the Faculty of Graduate Studies of
the University of Manitoba in partial fulfillment of the requirements
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FOR

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ABSTRACT

L-Ascorbic acid is an essential biological component, but its functional role in the living cell is not clearly understood. Ascorbic acid was shown to inhibit lysosomal hexosaminidase (Kanfer and Spielvogel, 1973) and β-glucuronidase (Dolbeare and Martlage, 1972). These observations prompted systematic studies to determine whether ascorbic acid was a regulatory molecule for lysosomal hydrolases.

In vivo studies employed guinea pigs whose diet was adequate in quantity of food and ascorbic acid (normal controls), scorbutic guinea pigs and guinea pigs pair fed to deficient animals but whose diet was adequate with respect to ascorbic acid. These studies showed that hexosaminidase, β-glucuronidase, α-galactosidase, α-mannosidase and acid phosphatase activities in liver homogenates from scorbutic animals were higher than activities found in homogenates from control groups. In brain homogenates, β-glucuronidase and acid phosphatase followed a pattern similar to that observed with the liver enzymes. Brain hexosaminidase was unaffected by ascorbic acid deficiency.

Subsequent studies with isolated hepatic lysosomes showed that the specific activities of hexosaminidase, β-glucuronidase, α-galactosidase, α-mannosidase and acid phosphatase from scorbutic guinea pigs were higher than those of normal and pair fed animals while cathepsins A, B, D and E, acid lipase, arylsulphatase A and B and β-glucosidase were similar to those of control groups.

Guinea pigs marginally deficient in ascorbic acid were used to see if the effects observed in scorbutic animals were specifically the results of ascorbic acid deficiency. Guinea pigs were fed an ascorbic acid deficient diet for 2 weeks and then a stock diet containing 0.5 mg ascorbic acid/g diet, or the deficient diet plus oral administration of 10 mg ascorbic acid/day or 1 mg or 0.5 mg ascorbic acid/100 g body weight/day for 10 weeks. Animals were periodically sacrificed and lysosomes isolated from individual livers. Serum and brain ascorbate declined when the vitamin was withheld, returned to normal when the stock diet or 10 mg ascorbic acid were fed but remained at low levels on administration of 1.0 mg or 0.5 mg ascorbic acid/100 g body weight. Brain norepinephrine

followed a similar pattern to brain ascorbate and was opposite to the pattern observed for dopamine. Guinea pigs receiving 0.5 mg ascorbic acid/100 g body weight/day were biochemically deficient in the vitamin as they had significantly lower concentrations of norepinephrine than control animals.

Serum hexosaminidase and lysosomal cathepsin A and B were unaffected by dietary treatment. In animals receiving 1 mg or 0.5 ascorbic acid/100 g body weight each day, lysosomal hexosaminidase and acid phosphatase activities at week 10, but not week 12, were significantly higher than activities found in the other groups.

Hexosaminidase activity in lysosomes prepared from normal, pair fed and scorbutic guinea pigs was used for in vitro studies examining the inhibition of the enzyme by ascorbic acid. Regardless of the group, the effects of ascorbic acid were similar. Physiological concentrations (2.5 mM) of ascorbic acid inhibited the enzyme somewhat whereas 70% inhibition could be obtained with 500 mM ascorbic acid. The inhibition was reversible and noncompetitive, but not totally specific for ascorbic acid since the D-isomer and araboascorbate also inhibited the

enzyme. Glucoascorbate and dehydroascorbate, were without effect as were various reducing agents and organic acids with pK's similar to ascorbic acid. Oxygen and metal ions were a prerequisite for ascorbate inhibition of hexosaminidase. Specific scavengers of O_2^-, $^1O_2$, OH. and H_2O_2 indicated that these species were not involved suggesting that monodehydroascorbate or some intermediate in the oxidation of ascorbic acid to dehydroascorbate was the inhibitory species.

Although the biochemical processes associated with the lysosomes are known, the underlying regulatory processes are still largely undefined and the involvement of ascorbic acid as a regulatory molecule for lysosomal enzymes is equivocal.

ACKNOWLEDGEMENTS

I am grateful to my thesis supervisor, Dr. Julian N. Kanfer, for his continual interest and advice in the direction of this research and in the preparation of this manuscript. I appreciate the efforts of Drs. Blanchaer, Dakshinamurti, Jamieson and Wang for their advice and interest.

Thanks are also extended to Dr. Johnson for his advice as to the statistics to employ in these studies. I am grateful to Dr. Hoogstraten for the electron micrographs.

I would like to acknowledge the receipt of a Medical Research Council of Canada studentship and a University of Manitoba Fellowship, which enabled me to carry out these investigations.

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ABBREVIATIONS

4MU	4-methylumbelliferone
4MU-GlcNac	4-methylumbelliferyl-β-D-N-acetyl-glucosaminide
AA	L-Ascorbic acid
hexosaminidase	β-D-N-acetylhexosaminidase (EC 3.2.1.52)
EDTA	disodium dihydrogen ethylenediaminetetraacetate dihydrate
EGTA	ethyleneglycol-bis(β-aminoethylether)-N, N'-tetraacetic acid

1. INTRODUCTION

Lysosomes were discovered in the early 1950's by deDuve and collaborators who observed an increase of detectable acid phosphatase activity in liver homogenates which had been refrigerated for several days. A series of investigations followed this observation and characterized lysosomes as sedimentable intracellular organelles containing hydrolytic enzymes with an acid pH optimum. Subsequently, the physiological function and pathological processes associated with lysosomes have been documented. However, little is known of how the lysosome and its complement of enzymes are regulated.

Few, if any, of the lysosomal hydrolases have cofactor requirements, and nor has it been demonstrated that they respond to allosteric effectors (Desnick et al., 1976). However, enzyme activity may be modulated by the presence of activators or inhibitors. Some activating factors, of lysosomal origin, have been described for cerebroside sulphatase (Jatzekewitz, 1978), glucocerebrosidase (Ho and Light, 1973) hexosaminidase A and α- and β-galactosidase (Li et al., 1974). The physiological role of these activators is far from clear, for it is not known why the enzymes are not activated with the subsequent

catabolism of the lysosome and the release of lysosomal enzymes into the cytoplasm which would result in cell death.

Some in vitro inhibitors of lysosomal hydrolases have been documented. For example, β-galactosidase and cerebroside glucosidase are inhibited by acidic oligosaccharides such as heparin and chrondroitin sulphate (Weinreb, 1976). Few, if any of the lysosomal enzymes have metal requirements (Desnick et al., 1976) however, lysosomal glycosidases are inhibited by mercurial compounds (Kanfer et al., 1972; Klibansky et al., 1974; Alhadeff et al., 1975) and Conchie and Levvy (1957) generalized that sugar lactones, but not the sugar acids, were capable of inhibiting the corresponding glycosidase.

One naturally occurring lactone is ascorbic acid (AA, vitamin C). AA has hydrolytic activity as a chemical reagent and the hydrolytic effects of the vitamin on plasmalogen (Yavin and Gatt, 1972), transferrin (Robinson et al., 1973), myelin basic protein (Westhall et al., 1976), phosphate esters (Maejima and Krescheckx, 1971) and 4-methylumbelliferyl-glycosides (Kanfer and Spielvogel, 1976) have been documented. At least for phosphate esters and

4-methylumbelliferyl glycosides, the products of the cleavage by AA are similar to those observed with lysosomal acid phosphatase or lysosomal glycosidases. Furthermore, AA can affect lysosomal enzyme activity.

AA inhibits partially purified hexosaminidase of bovine serum albumin (Kanfer and Spielvogel, 1972) and β-glucuronidase of lysosomal suspensions (Dolbeare and Martlage, 1972).

The similarity of these hydrolytic activites of AA to lysosomal enzymes and the inhibitory effects of AA on lysosomal hydrolases may merely be coincidental, but the possibility of the involvement of AA in lysosome enzyme regulation warrants investigation.

The purpose of this study is to focus on and examine the hypothesis that AA may be a physiological regulator of lysosomal enzyme activity. In vivo and in vitro experiments will be described to examine this hypothesis.

The animal model for these studies is the guinea pig which, like man, is dependent on a dietary source of AA (Burns, 1957). The deficient state (scurvy) is induced by feeding a commercially available diet devoid of AA, and the animals sacrificed when the clinical signs of scurvy

are apparent.[1] Measurement of AA tissue concentrations is used to confirm the AA status of the animal. For these experiments, a normal control group of guinea pigs receiving a diet adequate in quantity and nutrients, AA deficient animals, and a starved control group of guinea pigs pair fed to deficient animals but whose diet is adequate with respect to AA are required. The starved control group compensates for the anorexia observed with deficient guinea pigs.

However, the effects of acute AA deficiency on lysosomal hydrolases may not be due to a lack of AA per se. The effects may be secondary to some other effect of scurvy such as rapid weight loss or the general demise of the animal. Specificity may be demonstrated using guinea pigs chronically deficient in AA. In this model, tissue AA levels remain low and similar to those of scorbutic animals while growth proceeds normally without overt clinical signs of scurvy (Ginter et al., 1967; Ginter, 1970; 1977). This should eliminate other complications of scurvy such as haemorrhages, negative nitrogen balance and diarrhea.

[1] Loss of body weight, hair loss, paralysis, diarrhea.

To substantiate the in vivo significance of AA on lysosomal hydrolases, the possibility that AA is a small regulatory molecule will be investigated in vitro by examining the kinetics of AA inhibition of hexosaminidase, the effects of pH, AA analogues and the role of oxygen and metal ions. Hexosaminidase activity of isolated lysosomes will be used for these studies as the characteristics of the purified enzyme have been documented.

2. REVIEW OF LITERATURE

All mammalian cells, with the exception of erythrocytes contain lysosomes. Lysosomes represent a group of cytoplasmic organelles containing a variety of hydrolytic enzymes with acidic pH optima capable of breaking down proteins, nucleic acids, complex carbohydrates and lipids (Desnick et al., 1976). In vitro, the activity of these enzymes is latent and only fully expressed when the lysosomal membrane is broken by such procedures as osmotic shock, sonication, freezing and thawing or by the addition of detergents.

Significant progress has been made in understanding the functional and pathological processes of the lysosomes, but little is known of how the lysosomal enzymes are regulated.

Many physiological functions in the animal are affected by L-ascorbic acid (AA, vitamin C) and a large number of pathological lesions appear in AA deficiency. There are a number of reports of AA in maintaining the integrity of the cell structure. For example, connective tissue and the intercellular cement substance contain hydroxyproline and hydroxylysine and the role of AA in the

hydroxylation of proline and lysine residues of collagen has been documented (Barnes and Kodicek, 1972; Myllyla et al., 1978; Bates, 1979). Woodruff (1975) ascribed the pathological changes[1] observed in AA deficient animals secondary to changes in connective tissue and to changes in the integrity of the intercellular cement substance of the cells and capillaries. However, if lysosomal enzymes with the capacity to initiate digestion and catabolism are released from lysosomes into the cell to initiate cellular catabolism, subsequent release of these enzymes into extracellular spaces may be an important contributing factor in the decomposition of connective tissue.

Collagenase has been identified in lysosomes (Wynn, 1967). It is activated in scurvy induced in guinea pigs (Barnes and Kodicek, 1972) and the release of collagenase leads to catabolism of connective tissue. It has been suggested that the reduction in tissue AA brings about the digestion of mucopolysaccharides through the release of hyaluronidase, β-glucuronidase and β-galactosidase from

[1] Diarrhea, irritability, haemorrhaging, swelling over the long bones, pseudoparalysis, hair loss, anorexia, epistaxis

the lysosomes (Wilson, 1974).

Desai et al., (1964), Terroine and Hitier (1969) and Terroine (1971) postulated that AA was involved in the structural and numerical control of lysosomes in guinea pigs. The effect of AA deficiency on lysosomal enzymes of muscle and testicular tissue has been studied (Hitier, 1968; Hitier and Terroine, 1968).

Deficiency of AA in guinea pigs resulted in increased activity of acid phosphatase, DNase and RNase. These enzymes were presumed to be of lysosomal origin because of their acid pH optima. The increase in activity was apparent by the 15th day of AA depletion and continued to increase as the deficient state progressed. Degenerative changes in the muscle fibre were apparent by the 15th day of the experiment (Hitier, 1968a). It is at this time that weight loss commences (Ginter et al., 1967; Ginter, 1976). It was suggested (Hitier, 1968; 1968a; Hitier and Terroine, 1968), that the increase in acid hydrolase activity may have been due to increased number of lysosomes.

However, in scurvy, the muscle degenerates and becomes necrotic; haemorrhaging occurs and the influx of blood cells, especially macrophages which have a high