LYSOSOMES AND L-ASCORBIC ACID

by

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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.

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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

lysosome content (Gordon, 1973) could contribute to the increased activity. There is some evidence that this is so in the vitamin E deficient rat. In vitamin E deficiency, the muscle fibre degenerates and Zalkin et al., (1962) attributed the increase in lysosomal enzyme activity to the increased number of macrophages present in the tissue.

Alternatively, Wilson (1974) suggested that the increase in acid hydrolase activity observed in skeletal muscle of scorbutic guinea pigs may be an attempt by the tissue to regenerate. A periodic increase in lysosomal enzyme activity is known to occur in tissue undergoing active regeneration (Poole, 1973) which coincides with the cycle of mitotic division (Adams, 1963). Although Hitier (1968) and Terroine and Hitier (1968) found that acid phosphatase DNase and RNase activity continually increased from day 15 to 25 of their experiment, no data was given concerning the mitotic activity of the tissue.

The increased activity of acid phosphatase, DNase and RNase may be due to the loss of some inhibiting factor. AA has been shown to inhibit partly purified hexosaminidase of bovine serum albumin (Kanfer and Spielvogel, 1973) and β-glucuronidase of rat liver lysosomes (Dolbeare and Martlage, 1972). However, the occurrence of AA in

lysosomes has not been demonstrated and the physiological significance of AA inhibition of hexosaminidase and β-glucuronidase is not known at this time.

Other studies by Terroine and Hitier (1969) focused on the effects of AA depletion on lysosome stability. In vitro studies examined the differences between free activity of acid hydrolases in tissue homogenates and the total activities obtained after freezing and thawing. These authors found that the percent of free activity to total activity of acid phosphatase and acid DNase increased in AA deficient guinea pigs. It was suggested that the increase of free activity was due to increased lability of the lysosomal membrane possibly due to peroxidative damage.

Biomembranes and subcellular organelles are the major sites of lipid peroxidation damage and peroxidation of phospholipid unsaturated fatty acids which are part of the lysosomal membrane has been cited as a cause of lysosomal membrane labilization in vitro (Zalkin et al., 1962; Wills and Wilkinson, 1966; Wills, 1968). Lability and subsequent rupture of the lysosomal membrane with the concurrent release of lysosomal enzymes could account for

the increase of free activity observed by Terroine and Hitier (1969).

Studies on the effect of in vitro lipid peroxidation and free radicals on isolated lysosomes (Tappel, 1968) showed that these organelles underwent lipid peroxidation and that exposure of lysosomes to peroxidizing linoleate released lysosomal enzymes in direct proportion to peroxidative damage. In vitro AA was found to be a prooxidant (Tappel, 1974) and addition of low concentrations of AA in vitro caused rupture and release of hydrolytic enzymes from intact, isolated lysosomes. AA can donate electrons to a suitable donor such as molecular oxygen, with subsequent generation of hydroxyl radicals. Hydroxyl radicals have been shown to lyse lysosomes in vitro. During lysosome lysis peroxidation of lysosomal lipids was observed (Fong et al., 1973).

The exact role of AA in peroxidative membrane damage is far from clear. Cytoplasm from brain and liver was shown to contain a factor identified as AA which stimulated phospholipid peroxidation of subcellular particles (Sharma and Murti, 1968; 1976; Sharma, 1977). In studies of thiamine diphosphatase activity in freshly prepared, isolated

brain microsomes, physiological concentrations of AA caused a significant amount of lipid peroxide formation (Iwata et al., 1979). However, lipid peroxidation induced by AA may depend on AA concentration; high concentrations of AA may actually protect brain microsomes (Seregi et al., 1978; Bishayee and Balasubramanian, 1971) and liver microsomes (Hunter et al., 1964; Wills, 1968) against lipid peroxidation.

In vivo it is not known whether AA per se protects or may be a cause of peroxidation of biological membranes. AA has been postulated to be a biological reductant (Sebrell and Harris, 1972). Thus, when tissue AA concentrations are low, then peroxidation of membrane lipids could occur in a manner similar to that ascribed for vitamin E (α-tocopherol) (Bieri and Anderson, 1960; Zalkin et al., 1960). In vitro, vitamin E and AA react rapidly with organic free radicals and it is widely accepted that the antioxidant properties of these two vitamins are responsible in part for their biological activity (Bielski et al., 1971; Schuler, 1977). Tappel (1968) suggested that vitamin E and AA act synergistically, vitamin E acting as the primary antioxidant and the

resulting vitamin E radical reacting then with AA to regenerate vitamin E. Packer and coworkers (1979) have reported direct observation of this interaction in vitro, although it is not known if there is any interaction between vitamin E and AA in vivo.

Interaction between vitamin E and AA may be difficult to detect in vivo as under certain conditions ascorbate radicals can be enzymatically reduced back to AA by a NADH-dependent system (Schneider and Staudinger, 1965; Schulze et al., 1970) and thereby linked to NADH oxidation and a wide range of normal biochemical processes. Moreover, interaction between vitamin E and AA may account for the fact that levels of lipid peroxide higher than levels observed in control animals have not been detected in AA deficient (Zalkin et al., 1960; Bieri and Anderson, 1960) or vitamin E deficient animals (Billitterri and Raoul, 1965; Hitier, 1968).

AA may have important membrane actions other than peroxidation of membrane unsaturated fatty acids because of its reducing properties. A relationship between free to bound acid hydrolase and free to total sulphydryl groups in cultured synovial fibroblasts has been observed (Chayen et al., 1969). Inagaki (1970) showed that AA and cysteine inhibited a membrane bound $K^{+}Na^{+}$ATPase which may

be involved in ion transport through the plasma membrane. Furthermore, sulphydryl reagents were shown to react with membrane -SH sites (Tsen and Collier, 1960; Jacob and Jandl, 1962). This resulted in K^+ loss, Na^+ gain and ultimately cell lysis. Furthermore, Ignarro (1971) found that intact lysosomes were unstable in the presence of Na^+. Thus, AA may be able to affect the oxidation state of the membrane leading to increases in cytoplasmic Na^+. This may cause lysosomal membrane lysis.

Alterations in the membranes of lysosomes may be important in the pathogenesis of scurvy, but whether membrane changes occur as a consequence of AA deficiency is not known. It is not clear whether the increased activity observed with some lysosomal hydrolases in scorbutic animals is secondary to other manifestations of scurvy or the primary cause of degenerative changes observed in skeletal muscle and testicular tissue (Hitier, 1968; 1968a; Hitier and Terroine, 1968). In addition, it is not known how much cellular damage is initiated by the release of lysosomal enzymes into the cytoplasm of the cell although the release of lysosomal hydrolases from the cell appears to be destructive (Weissman, 1972).

AA is a cellular constituent. The increase of acid hydrolase activity in AA deficient animals and the in vitro inhibitory effects of AA on hexosaminidase (Kanfer and Spielvogel, 1973) and β-glucuronidase (Dolbeare and Martlage, 1972) may be coincidental occurrences. Alternatively, AA may be a small regulatory molecule for lysosomal enzymes.

3. GENERAL EXPERIMENTAL PROCEDURES

3.1 Materials

3.1.1 Animals

Male albino guinea pigs were used in all experiments. In experiments using animals acutely deficient in AA and for in vitro studies on the effects of AA on lysosomal hydrolases, guinea pigs of 100 - 120g were purchased from Biolab., St. Paul, Minneapolis, U.S.A. Guinea pigs of 175 - 200g were obtained from Canadian Breeding Labs, Quebec, Canada for studies using animals chronically deficient in AA.

3.1.2 Chemicals

4-Methylumbelliferyl-β-D-2-acetamido-2-deoxyglucoside 4-methylumbelliferyl-α-D-mannoside, 4-methylumbelliferyl-β-D-glucuronide, 4-methylumbelliferyl-α-D-galactoside, 4-methylumbelliferyl-β-D-glucoside, 4-methylumbelliferyl phosphate and 4-methylumbelliferyl oleate were obtained from Koch Light Ltd., Colnbrook, England and employed as substrates for hexosaminidase, α-mannosidase, β-glucuronidase, α-galactosidase, β-glucosidase, acid phosphatase and acid lipase respectively. The standard 4-methylumbelliferone was from the same supplier. Koch Light Ltd. also provided

the substrate, p-nitrocatechol sulphate dipotassium salt, and the standard, p-nitrocatechol used for the assay of arylsulphatase A and B. The dehydroascorbic acid (60% dehydroascorbate) used in these studies was also obtained from Koch Light Ltd. Dr. J.J. Burns (Hoffmann La Roche, Nutley, N.J.) kindly provided the D-ascorbic acid. Tiron (1, 2-dihydroxybenzene-3, 5-disulphonic acid disodium salt) was obtained from Eastman Kodak Co. (Rochester, N.Y.). All other chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

Catalase (EC 1.11.1., specific activity 26.602 μmole of H_2O_2 decomposition/min per mg), peroxidase (EC 1.11.1.7., specific activity 104 μmole of H_2O_2 decomposition/min per mg) and superoxide dismutase (EC 1.15.1.1., specific activity 12300 units/mg) were obtained from Miles Biochemicals (Elkhart, In.).

3.1.3 Guinea pig diet

Guinea pigs were maintained on a guinea pig diet, analyzed to contain 0.5 mg AA/g, Wayne Feeds, Allied Mills, Chicago, U.S.A.) or an AA free pelleted diet (ICN Pharmaceuticals Inc., Life Sciences Group, Cleveland, Ohio, U.S.A.) prepared to the specifications developed by

Reid and Briggs (1953). The latter was purchased in 50 pound lots and stored at 4°C until required.

3.2 Tissue Preparation

3.2.1 Serum

Blood was obtained by cardiac puncture or exsanguination and chilled on ice. The serum was separated at 4°C by centrifugation and stored in polyethylene vials at -20°C until required for analysis.

3.2.2 Liver subcellular fractions

Liver was removed immediately after sacrifice and subcellular fractions, including lysosomes were obtained by differential and gradient centrifugation (Ragab et al., 1967). Lysosomes were stored in distilled, deionized water at -20°C.

3.2.3 Brain

Brain was removed immediately after sacrifice and homogenized (1:8, w:v) in 0.9% NaCl. AA concentration was determined immediately, otherwise the tissue was stored at -20°C until required for analysis.

Due to the instability of tissue catecholamines, brains were removed immediately after sacrifice, frozen in liquid N_2, then wrapped in polyethylene sheeting, put

into amber bottles flushed with N_2 and stored at -50°C until required for analysis of dopamine and norepinephrine.

3.3 Assay Procedures

3.3.1 Lysosomal hydrolases

The reaction mixture for the determination of hexosaminidase, α-galactosidase, α-mannosidase, β-glucuronidase and β-glucosidase contained 0.25M citrate-phosphate buffer, pH 4.5, 50 µg bovine serum albumin free of the enzyme being studied, 0.125M NaCl, 2.5 mM substrate and guinea pig tissue homogenate or lysosomes in a total volume of 0.2 ml. For the assay of hexosaminidase, the bovine serum albumin was routinely purified (Kanfer and Spielvogel, 1977). For the assay of acid lipase, the reaction mixture contained 0.25M citrate-phosphate buffer, pH 4.4, 2.5 mM substrate in 0.25% Triton X100 and guinea pig lysosomes in a total volume of 0.2 ml. The reaction mixture for the assay of acid phosphatase contained 5 mM 4-methylumbelliferyl phosphate, 100 mM sodium acetate buffer, pH 4.5, and guinea pig tissue homogenate or lysosomes in a total volume of 0.2 ml. Each assay was carried out with three different amounts of protein in

duplicate. Tubes were incubated at 37°C for one hour and the reaction terminated with 2 ml of 0.25M glycine buffer, pH 10.3, and the 4-methylumbelliferone released was measured fluorometrically in an Aminco-Bowman spectrofluorometer (American Instrument Co. Inc., Maryland, U.S.A.) set at 366nm excitation and 446 nm emission (Raghavan et al, 1972).

Serum, liver homogenate and lysosomal hexosaminidase A were analyzed by a pH inactivation method (Perle and Saijer, 1975). Tubes containing 0.25 ml water or 0.25 ml 0.5M glycine buffer, pH 2.8 were incubated at 37°C. Duplicate aliquots of serum or liver homogenate or lysosomal protein were added, and incubated for 5 minutes at 37°C. Aliquots were transferred to tubes containing 0.25M citrate-phosphate buffer, pH 4.5 & 2.5 mM 4-methylumbelliferyl-β-D-2-acetamido-2-deoxy glucoside in a total volume of 0.2 ml. The tubes were incubated at 37°C for 30 minutes. The reaction was terminated by the addition of 2 ml of 0.25M glycine buffer, pH 10.3 and the 4-methylumbelliferone released measured fluorometrically. Specific activity was expressed as nanomoles of 4-methylumbelliferone released/ mg protein/hour.

Arylsulphatase A and B were estimated by a published procedure (Baum et al., 1959). For the assay of arylsulphatase A the reaction mixture contained lysosomal protein, 0.01M para nitrocatecholsulfate dipotassium salt and 0.5M sodium acetate-acetic acid buffer containing 5 mM $Na_4P_2O_7$, and 10% w/v sodium chloride, pH 5.0, in a total volume of 1.0 ml. The tubes were incubated at 37°C for one hour and the reaction terminated by the addition of 1.5 ml 1N NaOH. The tubes were centrifuged and the liberated para nitrocatechol in the supernatant determined by measuring the absorption at 515 nm against a water blank with a Gilford spectrophotometer. An appropriate control determination was also made in which the lysosomal protein and reagents were incubated separately and only mixed immediately before the addition of 1N NaOH.

Arylsulphatase B was determined in a similar manner as arylsulphatase A except that the 0.5M sodium acetate-acetic acid buffer was pH 6.0 and contained 100 mM barium acetate. Specific activity was expressed as nanomoles p-nitrocatechol released/mg protein/hour.

The method of Mycek (1970) was used for the assay of cathepsin D and E, cathepsin A and cathepsin B, using

bovine serum albumin as substrate. The incubation mixture for the assay of cathepsin D and E contained sodium citrate 5.7 mM with respect to citrate, pH 2.8, 5.7% bovine serum albumin and lysosomal protein in a total volume of 7 ml. For the assay of cathepsin A and cathepsin B, the incubation mixture contained 5.7 mM citric acid adjusted to pH 5.0 with NaOH, 5.7% bovine serum albumin and lysosomal protein in a total volume of 7 ml. As cathepsin B requires a sulphydryl activator, the reaction mixture for the assay of cathepsin B contained 10 mM cysteineHCl pH 5.0. Tubes were incubated at 38^{o}C for 10 minutes and the reaction stopped by the addition of 9 ml of 5% TCA. Denatured material was removed by filtering through a Whatman No. 3 filter paper and the clear filtrate read spectrophometrically at 280 nm against a suitable blank prepared by adding the TCA to a mixture of buffer and enzyme, mixing and then introducing the substrate. Specific activity was expressed as units of catheptic activity/mg lysosomal protein/10 minutes digestion. One unit of catheptic activity is the amount of enzyme which causes an increase of 0.001 units of absorbance at 280 nm/10 minutes digestion.

In studies where AA or AA analogues were added to

the incubation mixtures, the pH of these solutions was adjusted to pH 4.5 with 2.5 N KOH prior to their addition. Km and Ki values were obtained by the double reciprocal plot procedure of Lineweaver and Burke (1934). All values were adjusted for non-enzymatic cleavage of the substrate by AA and the effects of the various chemicals on enzymatic activity and on the fluorescent measurement were corrected.

3.3.2 Glucose-6-phosphatase

The method of Swanson (1955) was used for the estimation of glucose-6-phosphatase. Inorganic phosphate was determined by an established procedure (Fiske and SubbaRow, 1925). Glucose-6-phosphatase activity was expressed as mg inorganic phosphate released/15 minutes incubation/mg protein.

3.3.3 Cytochrome oxidase

Cytochrome oxidase activity was estimated by measuring oxygen consumption with a Gilford oxygraph (Gilford Instrument Laboratories Inc., Oberlin, U.S.A.) as established by Schnaitman et al., (1967). Activity was expressed as µmoles O_2 consumed/minute/g protein.

3.3.4 Ascorbic acid

The 2, 4-dinitrophenylhydrazine method of Roe (1961)

was used to estimate tissue AA concentrations.

3.3.5 Protein

Protein content was determined by the method of Itzhaki and Gill (1964) using bovine serum albumin as a standard.

3.3.6 Dopamine and norepinephrine

Frozen brain tissue was ground in a pestle and mortar with dry ice to ensure a homogenous sample and dopamine and norepinephrine were estimated fluorometrically by the trihydroxyindole method of Jacobowitz and Richardson (1978). Internal standards were used to estimate amine recoveries and appropriate corrections were made.

4. IN VIVO EFFECTS OF ACUTE ASCORBIC ACID DEFICIENCY ON GUINEA PIG LYSOSOMAL HYDROLASE ACTIVITIES

4.1 Introduction

It is believed that the degradation of cellular components and phagocytized material occurs in the lysosomes. However, little information is available regarding the regulation of lysosomal enzymes. Several reports have indicated that AA may influence the activity of some lysosomal hydrolases in vitro. AA was found to inhibit the partially purified enzyme from bovine serum albumin (Kanfer and Spielvogel, 1973) and β-glucuronidase of lysosomal suspensions (Dolbeare and Martlage, 1972). In vivo, pharmacological amounts of AA were shown to inhibit β-glucuronidase in a lepromatous patient (Matsuo et al., 1975).

The purpose of this study was to examine the effects of AA deficiency on various acid hydrolases of guinea pig liver, serum and brain as well as in isolated liver lysosomes of these animals.

4.2 Experimental procedures

4.2.1 Experimental protocol

Male albino guinea pigs were maintained on a commercial pellet diet. After one week, the animals were divided into three groups and an AA deficient pellet diet was fed to each group. Group 1 was the normal control group and these animals obtained the diet and drinking water supplemented with 1% AA ad libitum. Group 2 was the scorbutic group and received the diet and drinking water without AA ad libitum. Group 3 was pair fed to group 2 and was the starved control group. These animals received the quantity of feed consumed by their AA deficient litter mates the prior day. The drinking water for each group was changed twice each day.

Guinea pigs were weighed and food consumption recorded and adjusted daily.

When guinea pigs (Group 2) showed the typical signs of scurvy-weight loss, diarrhea, hair loss and poor locomotion, they were killed by cervical fracture and serum, liver and brain obtained and treated as described previously (section 3.2).

Serum, liver and brain AA concentrations were estimated (section 3.3.4) as well as serum hexosaminidase, liver hexosaminidase, β-glucuronidase, α-galactosidase,

α-mannosidase and acid phosphatase and brain hexosaminidase, β-glucuronidase and acid phosphatase (section 3.3.1). Lysosomes were isolated from individual livers of 16 normal control or 11 scorbutic or 6 starved control guinea pigs and pooled on an equal protein basis and the activities of various lysosomal enzymes assayed for (section 3.3.1).

4.2.2 Statistical analysis

The results were statistically evaluated by means of Student's t-test (Snedecor and Cochran, 1967).

4.3 Results

4.3.1 Weight

The weight of starved control animals closely paralleled that of scorbutic animals while that of normal controls continued at a more rapid rate (Fig. 1).

4.3.2 Ascorbic acid levels

There were lower amounts of AA in all tissues examined from scorbutic guinea pigs (Table 1). A comparison of the tissue AA concentration among the three groups indicates that concentrations of AA in starved control animals is intermediate between that of normal and scorbutic animals.

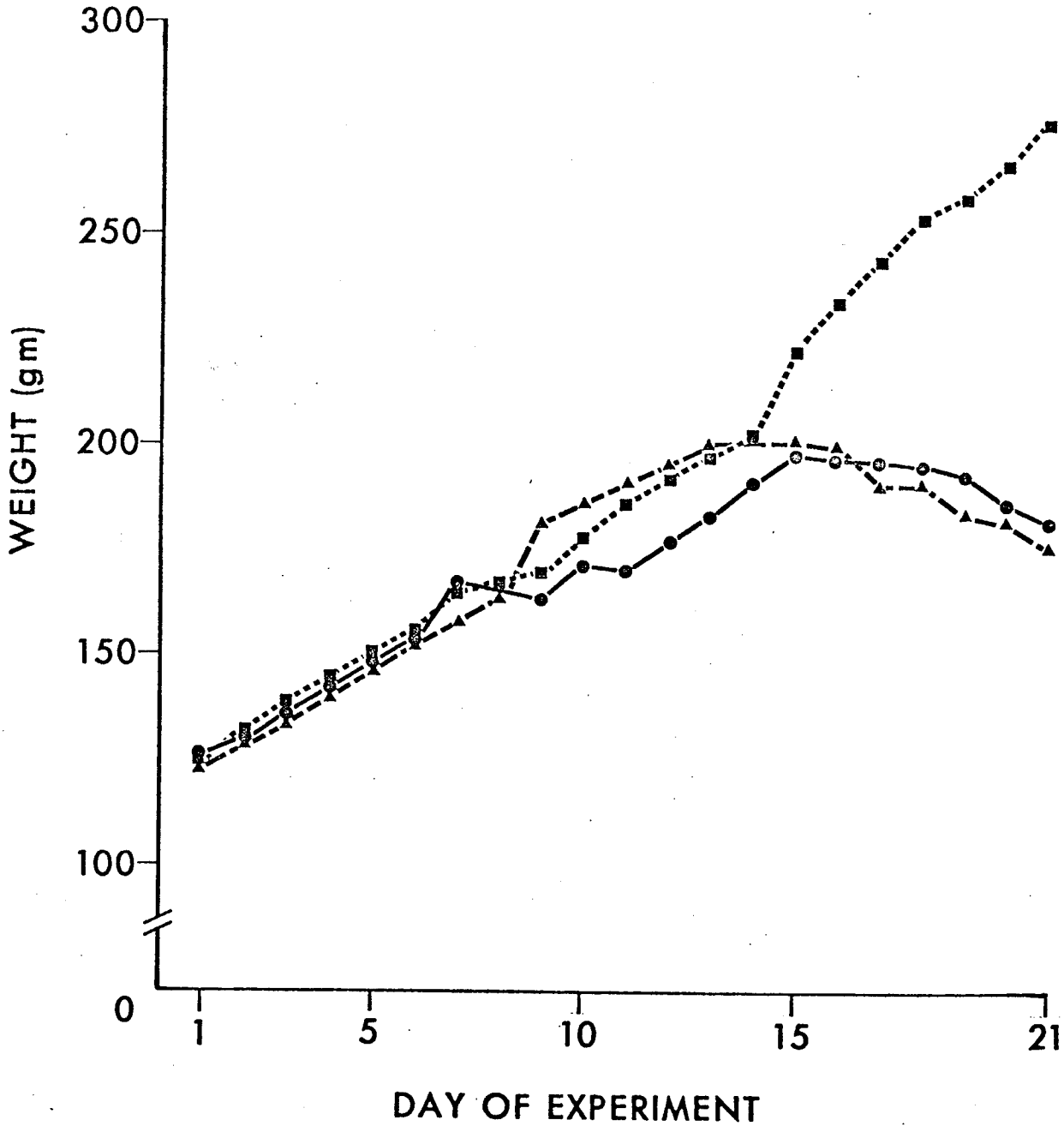

Figure 1. The weight of normal (■-----■), scorbutic (▲- - -▲) and starved (●——●) guinea pigs during acute ascorbic acid deficiency. The points represent the mean of 5 animals. The dietary regimen was changed on day 7 and the protocol is found in section 4.2.1.

Table 1

L-ascorbic acid concentration (mg per 100g wet weight tissue) in normal control guinea pigs, in starved control guinea pigs and in scorbutic guinea pigs [1]

Tissue	Normal Control	Scorbutic	Starved Control
Serum[2]	1.7 ± 0.1^{a}	0.3 ± 0.2^{b}	1.0 ± 0.1^{c}
Liver	37.7 ± 5.6^{d}	7.2 ± 1.1^{e}	20.6 ± 3.0^{f}
Brain	11.0 ± 2.4^{g}	4.8 ± 1.5^{h}	9.7 ± 1.4^{g}

[1] All values are expressed as the mean $\pm$ SD of 5 animals. Values not bearing the same superscript (a-h) are significantly different ($P < 0.05$).

[2] Expressed as mg/100 ml.

4.3.3 Effect of ascorbic acid deficiency on lysosomal hydrolase activity

The liver homogenates were assayed for hexosaminidase, β-glucuronidase, α-galactosidase, α-mannosidase and acid phosphatase activities and these results are shown in Table 2. Compared with normal control animals, these enzymes were greatly elevated in scorbutic animals and somewhat elevated in starved controls. Table 2 also depicts the activities of brain hexosaminidase, β-glucuronidase and acid phosphatase. No significant differences were noted between the groups for hexosaminidase, whereas β-glucuronidase and acid phosphatase activities followed a pattern similar to that observed with the liver enzymes.

Analysis of brain and liver AA concentrations showed that homogenates of tissues from normal and starved control animals contained less AA than that of normal control animals (Table 1). Therefore, the effect of adjusting the level of AA to the level present in normal control homogenates was examined for liver hexosaminidase (Table 3). The addition of AA had no measurable effect on the pH of the assay system. Under the conditions of AA supplementation, scorbutic and starved control

Table 2

Activity of several lysosomal hydrolases of guinea pig liver and brain homogenates[1]

Enzyme	Normal Control	Scorbutic	Starved Control
Liver			
Hexosaminidase	604 ± 53[a]	1798 ± 219[b]	1241 ± 379[c]
β-D-glucuronidase	163 ± 27[d]	465 ± 61[e]	298 ± 29[f]
α-D-galactosidase	17 ± 5[g]	88 ± 15[h]	39 ± 27[g]
α-D-mannosidase	19 ± 6[i]	64 ± 15[j]	29 ± 9[k]
Acid phosphatase	504 ± 132[l]	970 ± 123[m]	685 ± 266[l]
Brain			
Hexosaminidase	830 ± 250[n]	1106 ± 349[n]	1052 ± 97[n]
β-D-glucuronidase	36 ± 13[o]	66 ± 8[p]	45 ± 5[p]
Acid phosphatase	805 ± 211[q]	1274 ± 178[r]	1041 ± 187[s]

[1] All values are expressed as nmoles 4-MU released/mg protein/hour and represent the mean ± SD of 5 animals. Values not bearing the same superscript (a-s) are significantly different ($P < 0.05$).

Table 3

The effect of addition of L-ascorbic acid adjusted to the levels present in normal control animals on the activity of liver hexosaminidase[1]

	Scorbutic	Starved Controls
pmoles ascorbic acid added	360	250
- ascorbic acid	1657 ± 408	1069 ± 306
+ ascorbic acid	1496 ± 388	1028 ± 296
percent change	- 9.7*	- 3.9**

[1] All values expressed as nmoles 4-MU released/mg protein/hour and represent the mean ± SD of 5 animals.

* $P < 0.025$

** $P < 0.005$

hexosaminidase activity of liver homogenates decreased slightly (10% and 4% respectively).

Hexosaminidase can be separated into two predominant isoenzymes termed A and B. The effect of AA deficiency on the activity of these two enzyme forms present in guinea pig liver and serum was examined (Fig. 2). In normal guinea pig liver homogenates, hexosaminidase A comprised 51% of the total and increased to 55% in starved control animals. In scorbutic animals, hexosaminidase A accounted for 61% of the total activity. Hexosaminidase A of guinea pig serum followed a pattern similar to that observed in liver (45% in normal controls, 49% in starved controls and 52% in scorbutic animals).

Lysosomes were isolated from normal, scorbutic and starved control guinea pigs and were free of microsomal ($< 0.3\%$) and mitochondrial ($< 0.4\%$) contamination as judged by the activities of glucose-6-phosphatase and cytochrome oxidase which were used as markers for microsomal and mitochondrial contamination (Table 4). The specific activites of 12 lysosomal hydrolases were measured (Table 5). The specific activities of hexosaminidase, α-galactosidase, α-mannosidase,

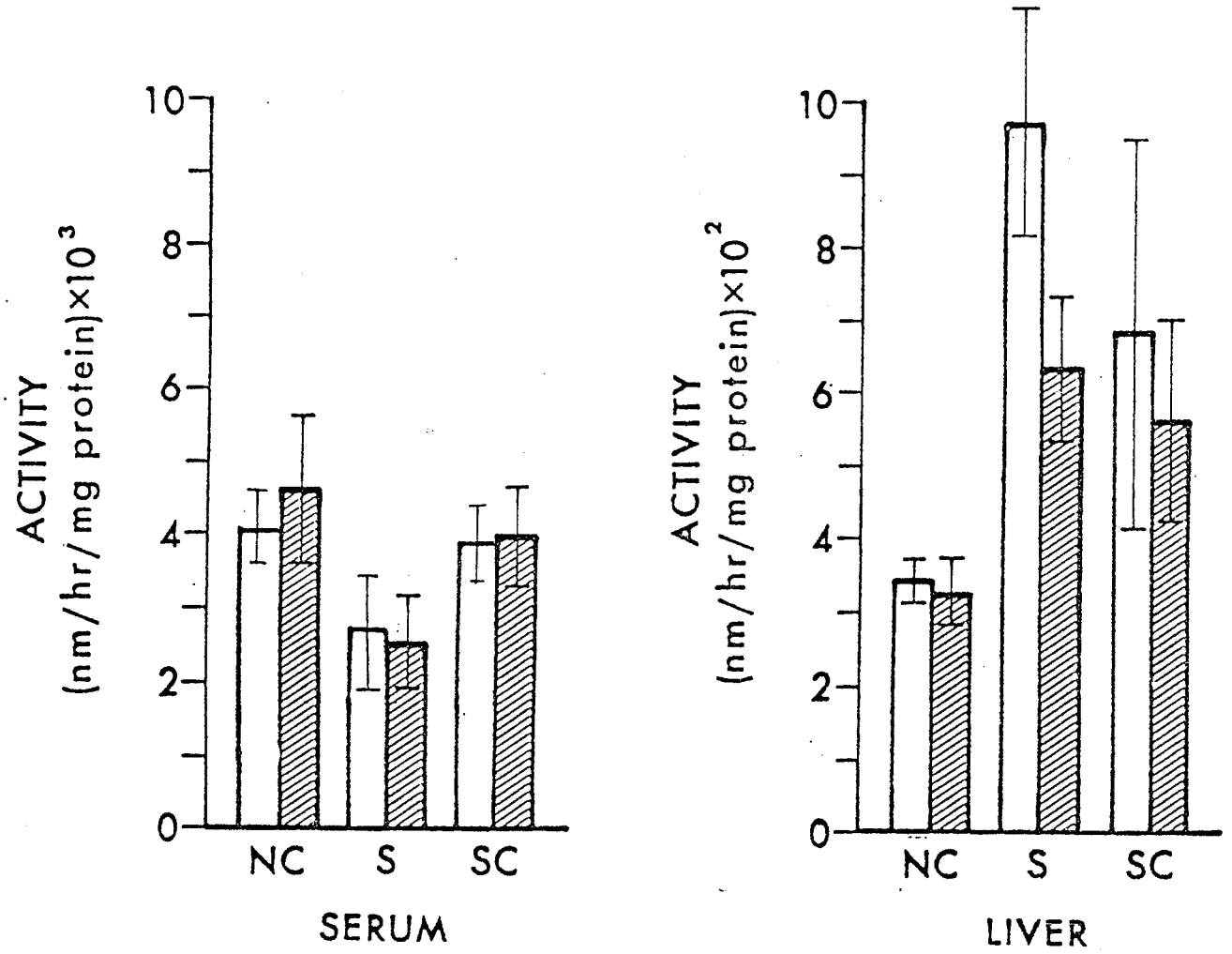

Figure 2. Activity of serum and liver hexosaminidase A and B isoenzymes. All values are expressed as nanomoles 4-methylumbelliferone released per milligram protein per hour and represent the mean ± SE of five animals except for serum where n = 4 for normal controls (NC), scorbutic (S) and starved controls (SC). Open bars represent hexosaminidase A and hatched bars represent hexosaminidase B.

Table 4

Total activities of glucose-6-phosphatase and cytochrome oxidase in liver homogenates and lysosomes from normal, scorbutic and starved control guinea pigs

	Normal Control	Scorbutic	Starved Control
Glucose-6-phosphatase[1]			
Liver homogenate	194.8	202.6	189.9
Isolated lysosomes	0.5	0.6	0.6
% contamination of lysosomes	0.3	0.3	0.3
Cytochrome oxidase[2]			
Liver homogenate	106.9	101.6	99.2
Isolated lysosomes	0.3	0.4	0.4
% contamination of lysosomes	0.3	0.4	0.4

[1] mg inorganic phosphate released per total amount of protein present in fraction.

[2] μmoles O_2/minute/total amount of protein present in fraction.

Table 5

Activity[1] of several lysosomal hydrolases of isolated guinea pig liver lysosomes[2]

Enzyme	Normal Control	Scorbutic	Starved Control
Hexosaminidase	1700	3280	1629
β-Glucuronidase	260	348	285
β-Glucosidase	30	34	29
α-Galactosidase	41	128	60
α-Mannosidase	110	280	137
Acid phosphatase	1332	2318	1457
Acid lipase	229	289	274
Arylsulphatase A[3]	445	581	379
Arylsulphatase B[3]	437	395	311
Cathepsin A[4]	301	218	265
Cathepsin B[4]	182	454	450
Cathepsin D & E[4]	1196	976	762

[1] Activity is expressed as nanomoles 4-MU released/mg protein/hour.

[2] Lysosomes from 16 normal, or 11 scorbutic or 6 starved guinea pigs were pooled on an equal protein basis.

[3] Using p-nitrocatechol sulphate.

[4] Units of catheptic activity/mg protein/10 min. One unit of catheptic activity is expressed as the amount of enzyme which causes an increase of 0.001 units of absorbance/minute digestion.

β-glucuronidase, acid phosphatase, arylsulphatase A, cathepsin B and acid lipase showed the following pattern: scorbutic were greater than normal controls which were similar to starved controls, although the magnitude of the elevation in the lysosomes isolated from scorbutic animals varied. For example, a 1.5 fold increase in specific activity was noted for β-glucuronidase, a 2 fold increase for hexosaminidase, α-mannosidase and acid phosphatase, whereas a 3 fold increase was noted for α-galactosidase. No differencesin specific activity were noted for arylsulphatase B, cathepsins D and E, cathepsin A and β-glucosidase.

The recoveries of protein, hexosaminidase and glucose-6-phosphatase and the specific activity of hexosaminidase are listed in Table 6. It is apparent that the total amount of hexosaminidase present in the subcellular fractions from the starved and control animals is approximately 50% of that present in the homogenates while in the scorbutic it is approximately 60%. There is a decreased amount of microsomal protein obtained from the experimental groups as compared with the controls and an increased quantity of lysosomal protein in the scorbutic group.

TABLE 6: RECOVERIES AND ACTIVITY OF SUBCELLULAR FRACTIONS FROM LIVERS OF CONTROL, STARVED, AND SCORBUTIC GUINEA PIGS

	Controls					Starved					Scorbutic				
	Protein		Hexosaminidase		Glucose 6-(P)-ase	Protein		Hexosaminidase		Glucose 6-(P)-ase	Protein		Hexosaminidase		Glucose 6-(P)-ase
	mg/g wet weight tissue	total g	SA[1]	Total Activity[2]	Total Activity[3]	mg/g wet weight tissue	total g	SA	Total Activity	Total Activity	mg/g wet weight tissue	total g	SA	Total Activity	Total Activity
Whole homogenate	330.1(13)[4]	4.05	635	2573	58.9(4)	287.4(8)	2.67	1245	3324	50.0(3)	199.2(13)	2.03	1659	3367	95.8(4)
Nuclear & Debris	40.7(8)	0.50	1373	687	35.1(4)	24.8(3)	0.43	2084	896	19.3(3)	39.3(8)	0.40	2902	1161	27.3(4)
Mitochondria (Mit)	4.9(8)	0.06	2227	134	0.3(4)	2.2(3)	0.03	2792	84	0.3(3)	3.9(8)	0.04	3765	151	2.5(4)
Light mitochondria	9.0(6)	0.11	1212	133	0.6(4)	1.1(3)	0.09	2219	200	0.1(3)	2.0(8)	0.09	2686	242	0.4(4)
Lysosomes (Lys)	1.6(8)	0.02	1970	39	0.3(4)	1.1(3)	0.02	2563	51	0.1(3)	4.9(8)	0.05	3681	184	0.2(4)
Microsomes	7.3(8)	0.09	808	73	12.9(4)	2.2(3)	0.06	1343	81	9.6(3)	2.0(8)	0.06	1549	93	14.4(4)
Lys/Mit composite	1.6(4)	0.02	2051	41	0.2(4)	1.1(3)	0.01	1904	19	0.1(3)	1.0(8)	0.01	3295	33	0.2(4)
Cytosol	67.6(8)	0.83	120	100	5.7(4)	59.2(3)	0.55	167	92	3.7(3)	79.5(8)	0.71	105	75	4.4(4)
1st 11700 x g supernatant	47.4(4)	0.58	111	64	6.9(4)	51.7(3)	0.48	134	64	2.8(3)	53.0(8)	0.51	175	89	12.5(4)
2nd 11700 x g supernatant	48.9(4)	0.60	24	14	2.7(4)	61.4(3)	0.57	21	12	1.5(3)	58.9(8)	0.60	39	23	2.5(4)
27000 x g supernatant	22.8(4)	0.28	26	7	0.4(4)	18.3(3)	0.17	29	5	0.2(3)	27.5(8)	0.21	45	10	0.7(4)
Total recovered		3.09		1292	65.5		2.41		1504	37.7		2.68		2061	65.1
% Recovered		76		50	111		90		45	75		132		61	68

[1] Specific activity is expressed as nanomoles 4 MU released/mg protein/hour
[2] Expressed as µmoles 4 MU released/hour
[3] Expressed as mg phosphate released/10 mins
[4] Represents number of animals

4.4 Discussion

In AA deficiency, there were decreased concentrations of AA in all tissues examined (Table 1). However, decreased tissue AA concentrations were observed in starved control animals. Presumably, these animals did not drink as much as those receiving unrestricted quantities of food. Alternatively, reduced metabolism resulting from decreased intake of nutrient material decreases tissue AA (Davies and Hughes, 1977). It has been postulated that differences in metabolic rate could explain the negative correlation of AA concentrations with age in man and guinea pigs and also the increase of tissue AA associated with protein-induced increases in growth rate (Andrews and Brook, 1966; Brook and Grimshaw, 1968; Hughes and Jones, 1971; Williams and Hughes, 1972).

A general increase of activity of several acid hydrolases in the tissue of scorbutic guinea pigs was demonstrated. This appears independent of inanition which is a characteristic of scurvy (Table 2) as body weights of starved and scorbutic animals were not greatly different (Fig. 1). The results of mixing experiments with homogenates of normal liver and those of scorbutic or

starved control animals indicates that no inhibitor or activator) is present.

A more detailed examination of the activities of some enzymes present in isolated lysosomes, indicated that in lysosomes isolated from deficient animals, the specific activities of several enzymes are elevated (Table 5). This was not observed for lysosomes isolated from pair fed animals. An explanation for this phenomenon is not readily available. There may be increased synthesis of the enzyme or decreased degradation of the enzyme. Alternatively, the enzyme may be more active towards the substrate.

The observations regarding the recoveries of proteins and enzymes presented in Table 6, suggest certain changes in the livers of deficient animals. It appears that the mass of material believed to be responsible for protein synthesis, the microsomes, is reduced. This is accompanied by an increase of lysosomal protein suggesting that there are more lysosomes of sufficient density to sediment in the system employed. There is no explanation for the poor recovery of hexosaminidase as combination and reconstitution experiments do not indicate the presence of activators or inhibitors.

Changes in the activities of some acid hydrolases have been reported in tissue homogenates from AA deficient guinea pigs. Increased acid phosphatase of guinea pig skeletal muscle (Hitier, 1968) and testicular tissue and deoxyribonuclease(Hitier, 1967) and cathepsin (Wilson, 1974) activity of liver and spleen homogenates has been noted. Collagenase is higher in scurvy (Wynn, 1967; Barnes and Kodicek, 1972) and it was suggested that the reduction in tissue AA brings about the degradation of mucopolysaccharides through the release of hyaluronidase, β-glucosidase and α-galactoside from lysosomes.

In one study (Terroine, 1971), electron micrographs of testicular tissue from scorbutic guinea pigs showed increased numbers of lysosomes. However, electron micrographs of liver and brain tissue from normal, scorbutic and starved control animals showed no discernable differences in the number of lysosomes between the groups (Plates 1, 2 and 3, Appendix 7).

It may be that AA influences the activity of lysosomal hydrolases as the addition of the vitamin comparable with the level present in normal control tissue decreased hepatic hexosaminidase activity somewhat but not the brain enzyme (Table 3). Although the liver and brain contain

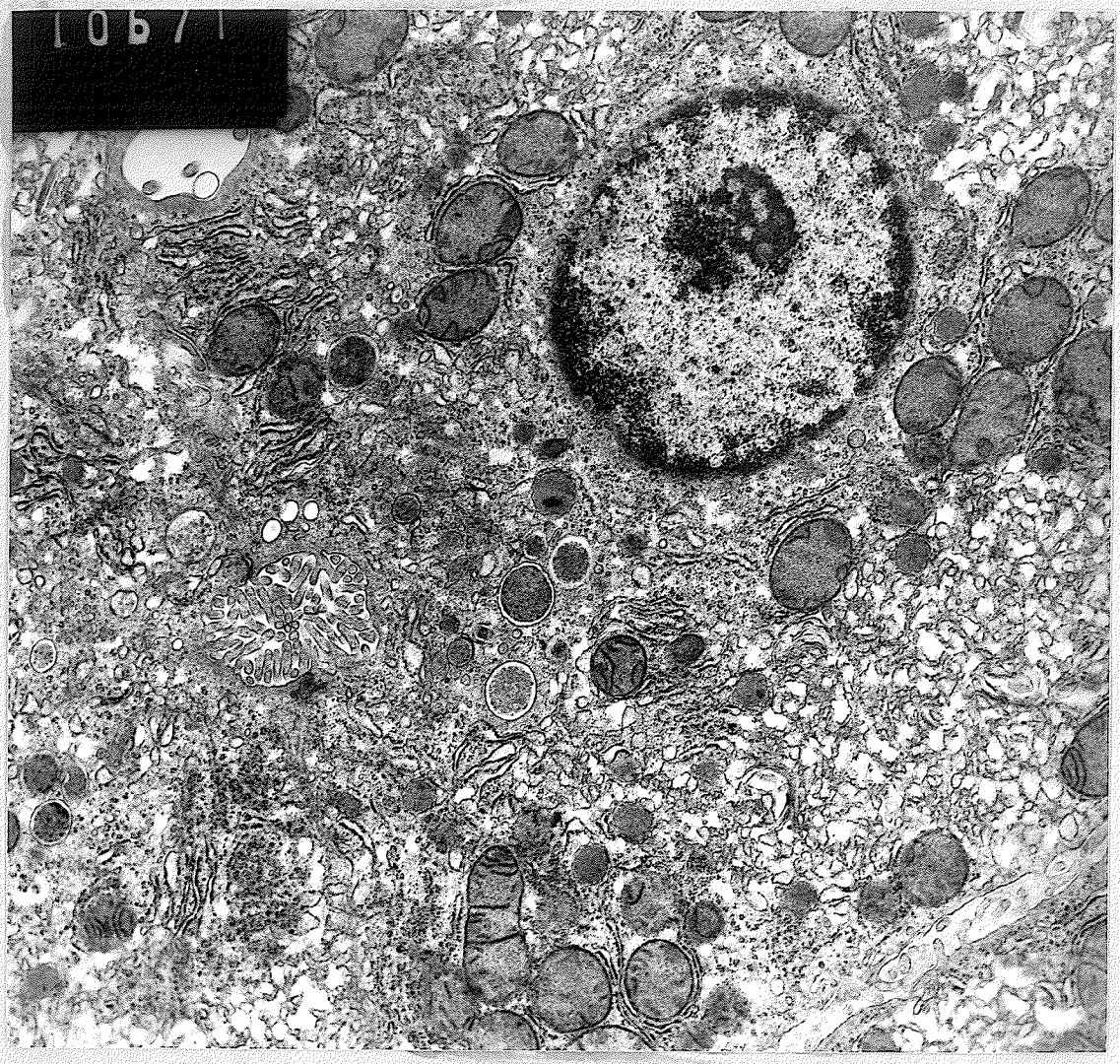

Plate 1: Electron micrograph of hepatocyte from normal control guinea pig. x 18,000

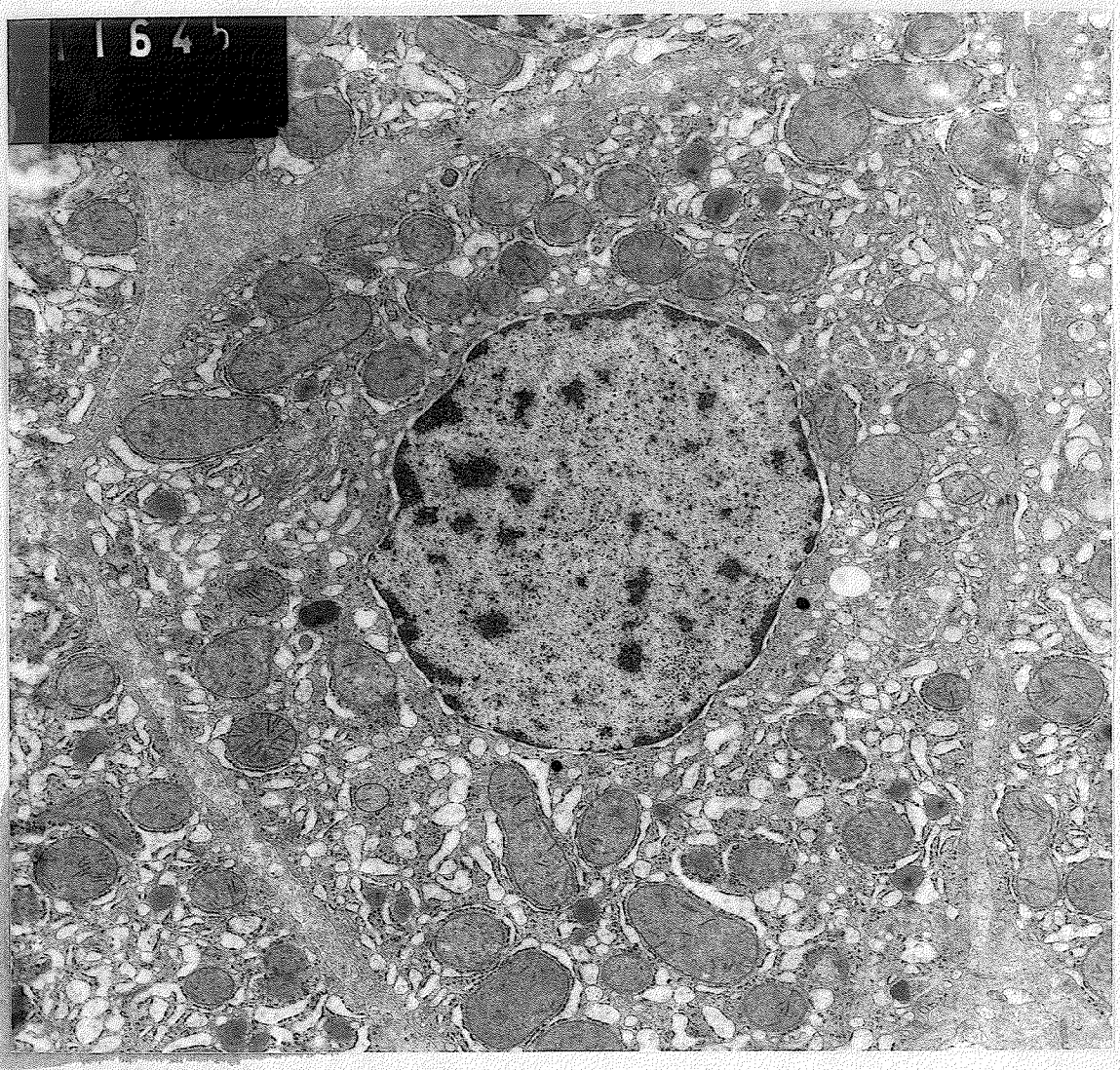

Plate 2: Electron micrograph of hepatocyte from scorbutic guinea pig. x 18,000

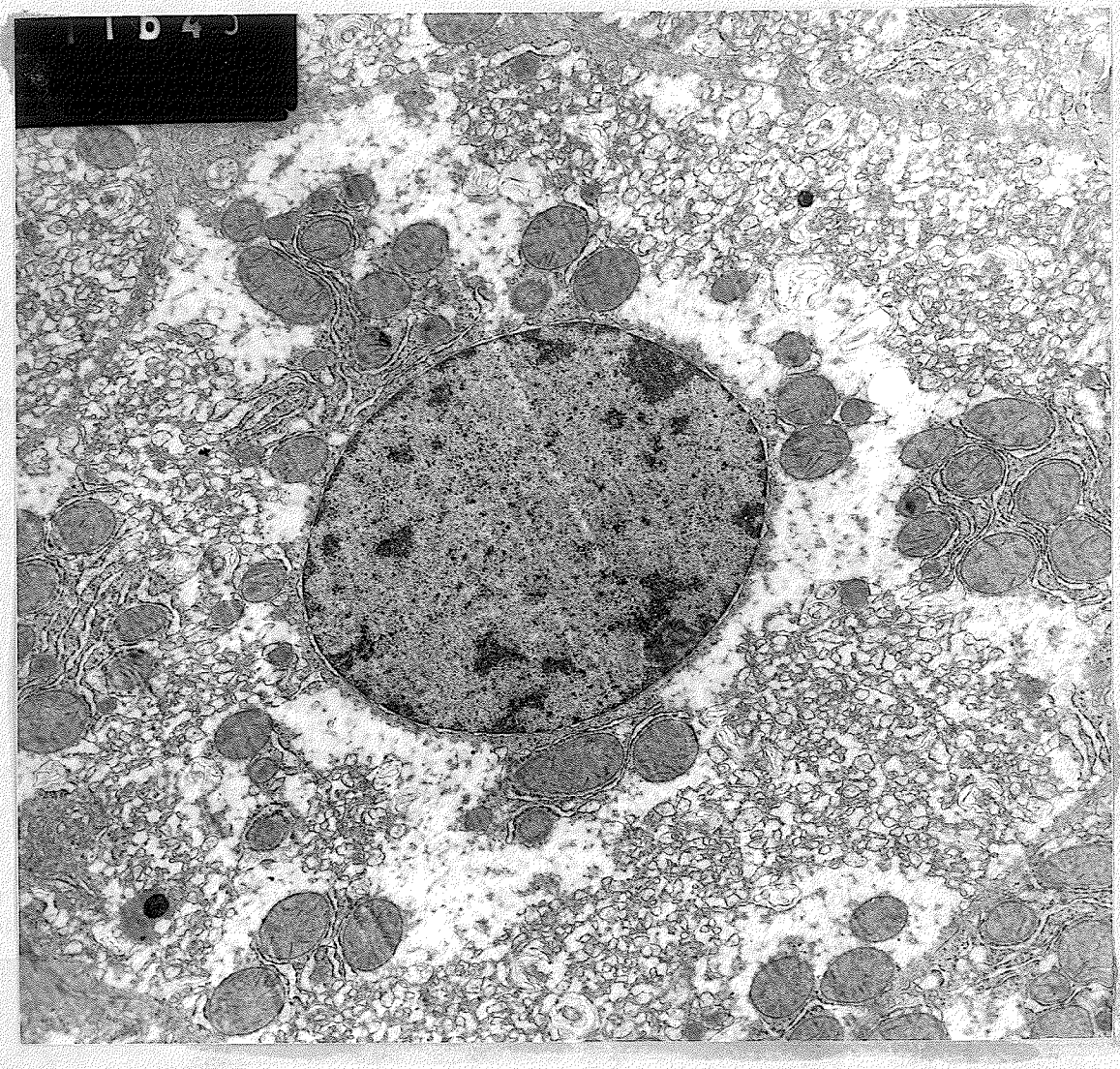

Plate 3: Electron micrograph of hepatocyte from starved control guinea pig. x 18,000

high concentrations of AA, it is not known whether the lysosomes contain the vitamin. Inhibition of β-glucuronidase (Dolbeare and Martlage, 1972) and partly purified hexosaminidase (Kanfer and Spielvogel, 1973) was demonstrated. The effect may be on the enzyme itself or on another component. Low molecular weight activators of sphingolipid hydrolases have been reported (Li et al., 1974; Mraz et al., 1976). In addition, in vitro the mucopolysaccharides are inhibitory for β-galactosidase (Kint et al., 1973) although inhibition is prevented by albumin (Kint, 1974) and chloride ions (Ho and Fluharty, 1975).

Scurvy and starvation did not affect serum hexosaminidase activity (Fig. 3) although the concentration of AA was diminished. It was suggested that in scurvy, the lysosomal membrane is more fragile (Terroine and Hitier, 1969). This appears not to be so in the present study, as the specific activity of hexosaminidase in the cytosol (Table 6) and serum of scorbutic animals was not increased.

In guinea pig liver (but not serum) the proportion of hexosaminidase A increased (Fig. 2). There are several

conditions in man where the proportion of hexosaminidase A and B isoenzymes change. Tay-Sachs disease is characterized by deficient activity of hexosaminidase A (Okada and O'Brien, 1969) and Sandhoff-Jatzkewitz disease by deficient activity of both hexosaminidase A and B (Sandhoff et al., 1971). In patients with diabetes mellitus, hexosaminidase B is elevated while hexosaminidase A and I increase during pregnancy (Nakagawa et al., 1977).

Although scurvy is characterized by decreased AA concentrations, interpretation of the experimental results is complicated by factors other than AA deficiency, such as lowered food consumption, abrupt loss of body weight and negative nitrogen balance. Although the specific activities of various enzymes of lysosomal origin are increased in tissues of deficient animals and in isolated hepatic lysosomes, these increases have only been described in severely deficient animals, and thus could be of a secondary character. For instance, the increase of lysosomal specific activity may be due to the general demise of the scorbutic animal resulting from a need for increased tissue catabolism as is observed in starvation

(Desai, 1969; 1971). There are other situations where increased activities of lysosomal enzymes are observed, such as during inflammation (Weissmann, 1972), atherosclerosis (Wolinsky and Fowler, 1978), cancer (Allison, 1969), chlorphentermine induced lipidosis (Lullmann and Mosinger, 1979), vitamin B6 (Diapaolo et al., 1975) and vitamin E (Zalkin et al., 1962) deficiency and excess vitamin A (Dingle et al., 1966). Even in normal situations such as development (Raychandhuri and Desai, 1972) and aging (Asano et al., 1979) changes are observed with some lysosomal enzymes. Thus, before the changes observed in lysosomal enzyme activity in scorbutic guinea pigs can be ascribed to a deficiency of AA per se, other factors such as the deterioration of the animal must be controlled.

5. IN VIVO EFFECTS OF CHRONIC ASCORBIC ACID DEFICIENCY ON GUINEA PIG LYSOSOMAL HYDROLASE ACTIVITIES

5.1 Introduction

The effects of acute AA deficiency were studied (section 4) and the elevated specific activities of several acid hydrolases of guinea pig liver and brain (Table 2), as well as increases in specific activities of hexosaminidase, α-galactosidase, α-mannosidase and acid phosphatase of isolated lysosomes were noted (Table 5). However, the increases in specific activity of these lysosomal enzymes could be of a secondary character due to the general demise of the animals rather than a specific decrease in tissue AA.

The aim of the present study was to use guinea pigs marginally deficient in AA, where there is no general inanition and no overt clinical signs of scurvy (Ginter et al., 1967), to see if the effects observed in severely AA deficient guinea pigs (section 4) were the specific results of AA deficiency.

5.2 Experimental procedures

5.2.1 Experimental protocol

Male albino guinea pigs with an initial weight of 175 - 200g were maintained on guinea pig diet for one week and then on specific diets. Water and food were allowed ad libitum. Animals were housed individually for the entire length of the experiment in stainless steel, wire bottomed cages equipped with an automatic watering system. Weights were recorded three times a week.

At the start of the experiment, week 0, the feed was changed to an AA free pelleted diet. A two week period served to deplete the body stores of AA. The animals were then randomly divided into four groups. A control group of animals (Group 1) were fed the guinea pig diet which was analyzed to contain 0.5 mg AA/g diet while Groups 2, 3 and 4 received the AA deficient diet with various oral supplements of AA each day. Group 2 received 10 mg AA each day; Group 3 received 1 mg AA per 100g body weight and Group 4 received 1 mg AA per 100g body weight for two weeks which was then reduced to 0.5 mg AA per 100g body weight. The AA was dissolved in 20% sucrose solution just prior to being administered orally. The

dosage volume was 100 µl/100g body weight.

The experiment was continued for 12 weeks with the exception of those in Group 1 where the experiment was terminated after 10 weeks. Food was withheld from the animals 12 hours prior to sacrifice and 2 or 4 animals from each group were killed by decapitation at weekly intervals and blood collected by exsanguination

Serum, brain and hepatic lysosomes were obtained and treated as previously described (section 3.2).

Serum hexosaminidase A and B, lysosomal hexosaminidase, acid phosphatase and cathepsins A and B were assayed for (section 3.3.1) as were brain norepinephrine and dopamine (section 3.3.6) and tissue AA (section 4.3.4).

5.2.2 Statistical analysis

Enzyme specific activities and brain dopamine and norepinephrine were assessed by analysis of variance to study treatment (diet) and time effects (Snedecor and Cochran, 1967). Because of the missing data (Group 1) and the unequal variance at week 12 for hexosaminidase and acid phosphatase, this period was evaluated using a one way analysis of variance and Welchs' approximation for unequal variance (Brownlee, 1960). Interactions between

treatments and time were studied for hexosaminidase and acid phosphatase by testing for polynomial comparisons.

5.3 Results

5.3.1 Growth

Growth expressed as weight gain showed similarity between the groups throughout the duration of the experiment (Fig. 3). At the end of the experiment, the g average body weight $\pm$ SD was 540 $\pm$ 49, 581 $\pm$ 44, 549 $\pm$ 47 and 569 $\pm$ 47 for groups 1, 2, 3 and 4 respectively.

5.3.2 Brain and serum ascorbate

The AA concentrations in serum and brain are presented in Figs. 4 and 5 respectively, and illustrates that the relative pattern of response of these two tissues was similar. During the depletion period (week 0 - 2), the AA concentration in brain and serum decreased. This effect was immediately reversed when the stock diet was fed (Group 1) or when 10 mg AA per day was administered (Group 2), although the recovery was slightly lower in the animals receiving 10 mg AA per day than those values observed from animals in Group 1. AA concentrations remained lower when 1 mg of the vitamin per 100g body weight was administered and was similar to that during the

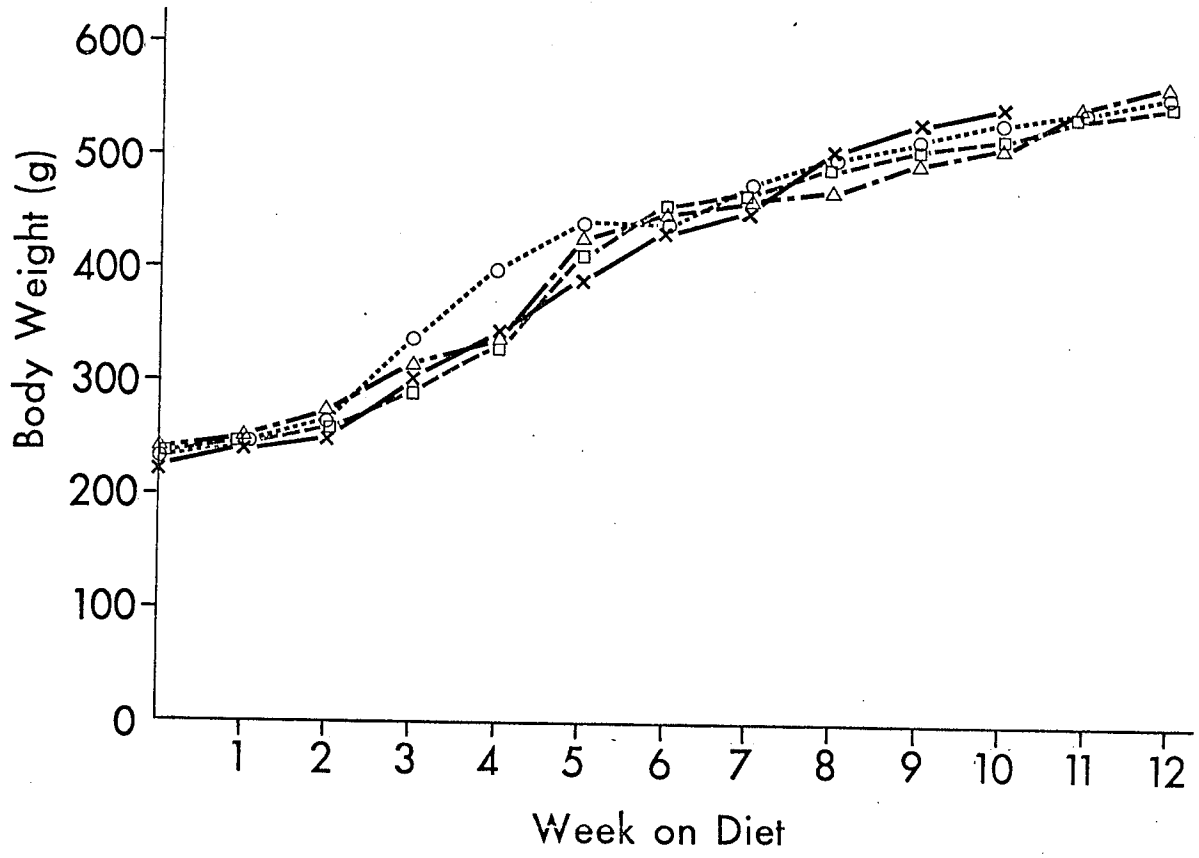

Figure 3. Weights of guinea pigs with various ascorbic acid intakes. In order not to overload the figure SD are omitted. During weeks 0-2, no AA was supplied; then animals received a stock diet containing 0.5 mg AA/g diet (X——X); 10 mg AA/day (□····□); 1 mg AA/100g body weight/day (O---O); 0.5 mg AA/100g body weight/day (△····△).

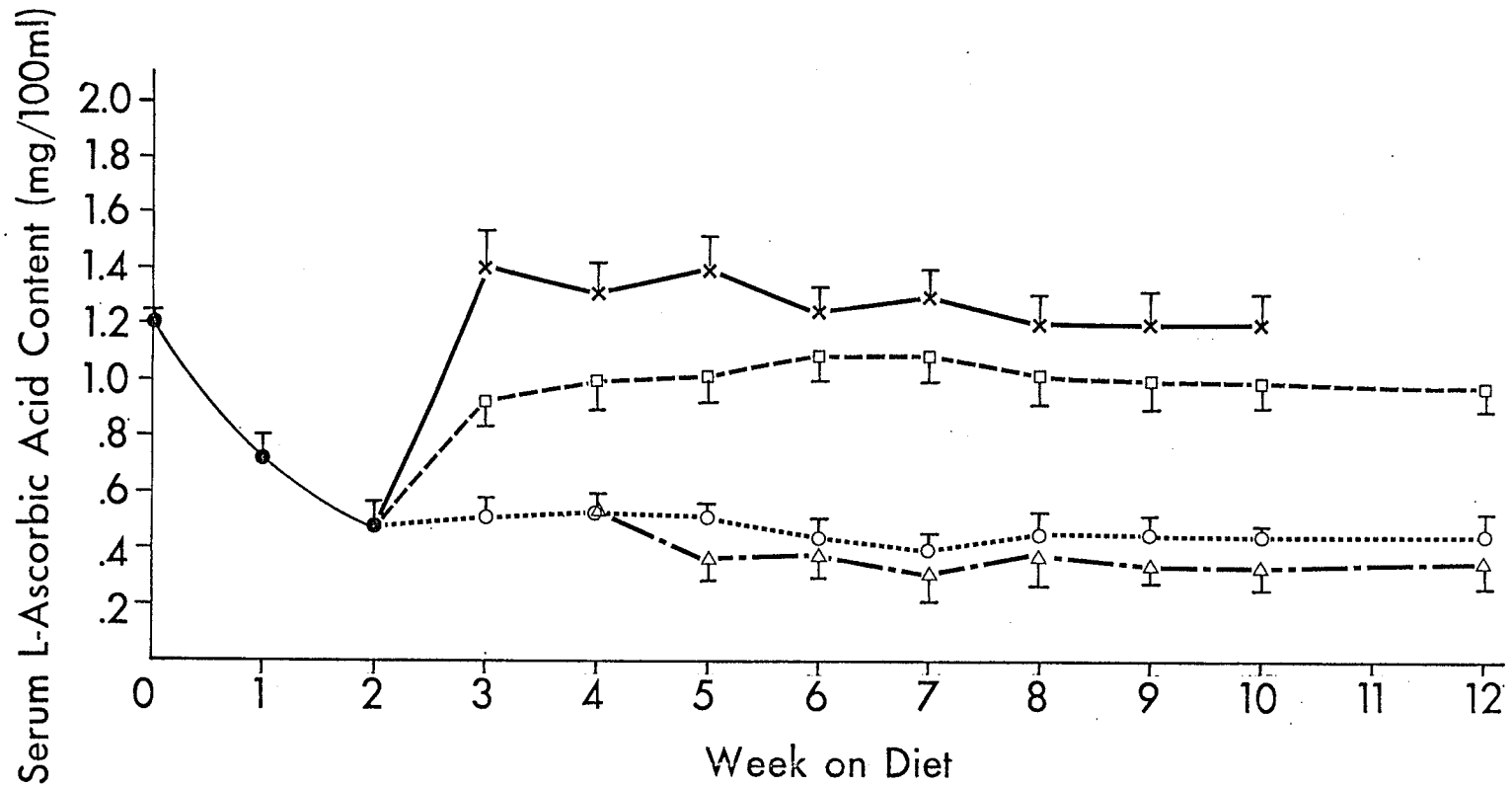

Figure 4. Ascorbic acid concentrations (mean ± SD) in the serum of guinea pigs fed various amounts of ascorbic acid for 12 weeks. During weeks 0-2, no AA was supplied (●——●) then animals received a stock diet containing 0.5 mg AA/g diet. (X——X); 10 mg AA/day (□---□); 1 mg AA/100g body weight/day (0----0); 0.5 mg AA/100g body weight/day (△---△).

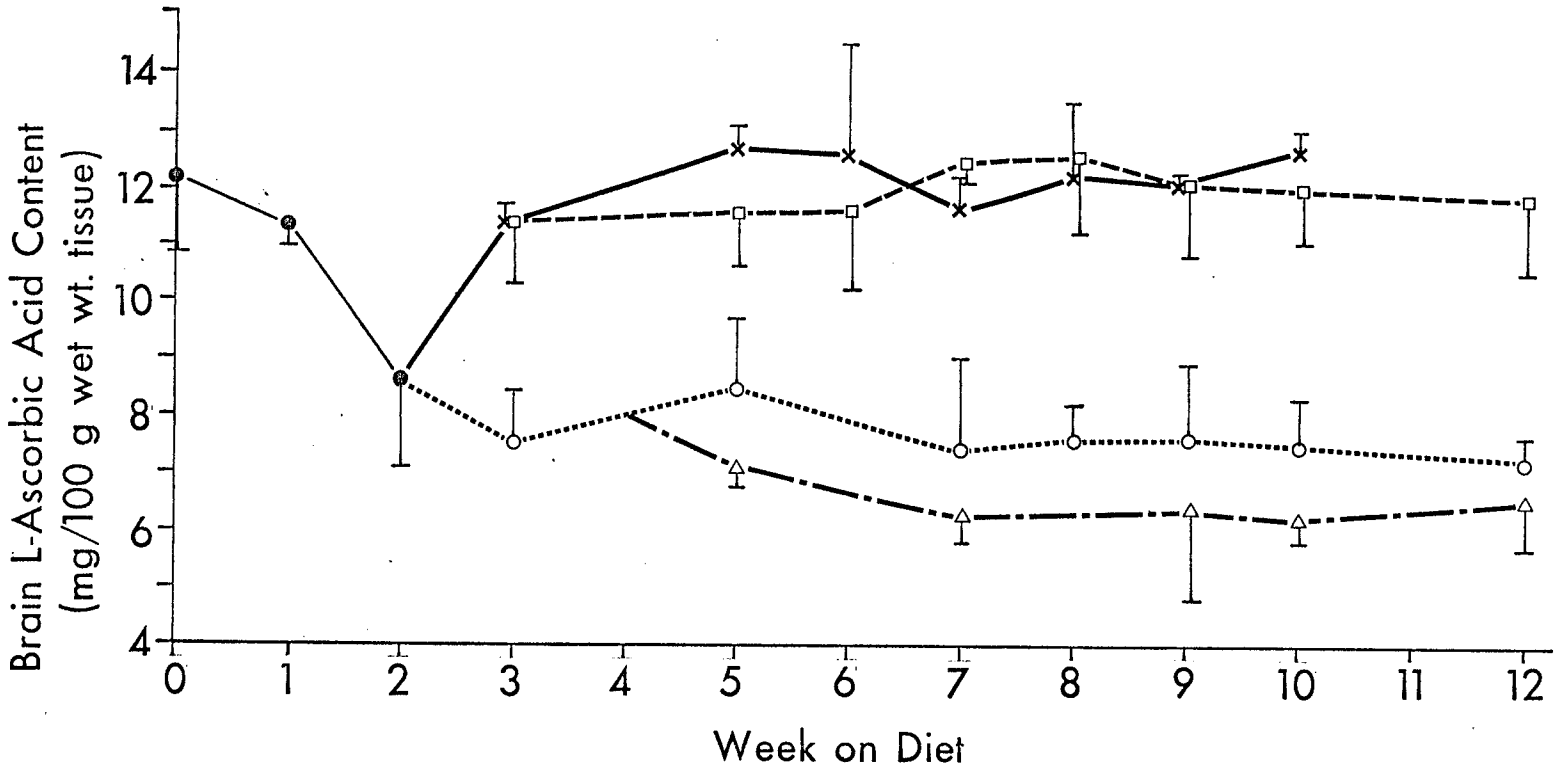

Figure 5. Ascorbic acid concentration (mean ± S.D.) in the brain of guinea pigs receiving various amounts of ascorbic acid for 12 weeks. During weeks 0-2 no AA was supplied (●——●) then animals received a stock diet containing 0.5 mg AA/diet (X——X) 10 mg AA/day (□--□); 1 mg (○---○) or 0.5 mg (△—·△)/100g body weight/day.

depletion period. When the amount of vitamin was reduced by half (Group 4) a further decrease of AA was observed in the brain and serum of these animals.

5.3.3 Enzyme activities

(a) Serum

The effect of the degree of marginal AA deficiency on the activity of serum hexosaminidase as well as the two predominant hexosaminidase isoenzymes, A and B, was examined. No differences were noted between the groups for total hexosaminidase although those animals receiving 10 mg AA/day (Group 2) tended to have a higher specific activity of this enzyme (Fig. 6). The relative proportions of hexosaminidase A and B remained constant throughout the experiment, hexosaminidase A accounting for 50 - 60% of the total hexosaminidase activity of the serum.

(b) Lysosomes

No differences were noted between the groups for cathepsin A and cathepsin B (Fig. 7, Appendix 1 and 2). However, for hexosaminidase (Fig. 8, Appendix 3), there was a significantly different response to the dietary treatment ($P < .001$) between week 5 and 10 which was not observed at week 12. In general, Groups 3 and 4 which received the lowest concentrations of AA had the highest specific activity; the converse was

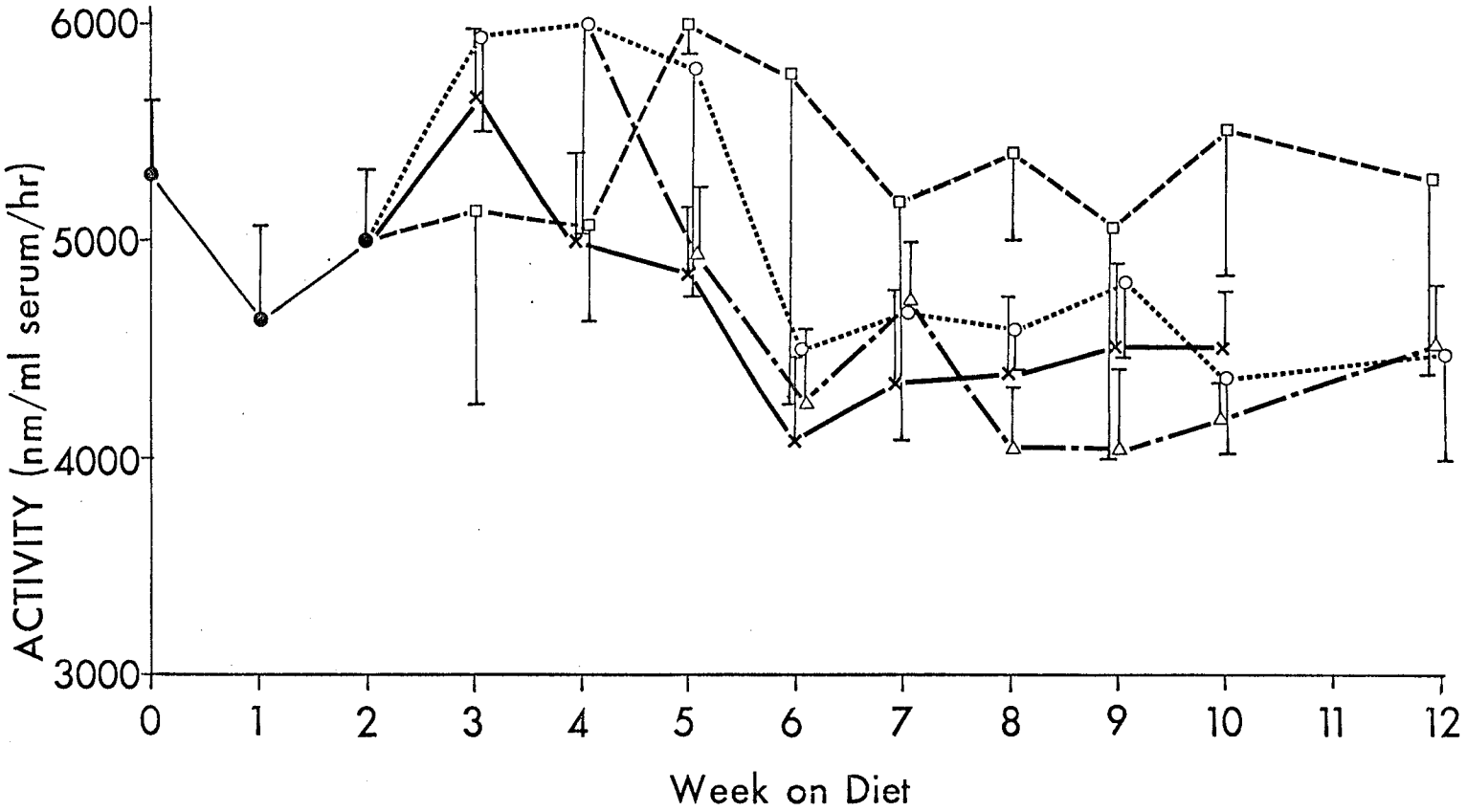

Figure 6. Activity of serum hexosaminidase of guinea pigs receiving various amounts of ascorbic acid for 12 weeks. All values are expressed as nanomoles 4-MU released per ml serum per hour and represent the mean ± SD. During weeks 0-2 no AA was supplied (●——●) then animals received a stock diet containing 0.5 mg AA/diet (X——X); 10 mg AA/day (□--□); 1 mg (O---O) or 0.5 mg (△--△)/100g body weight/day.

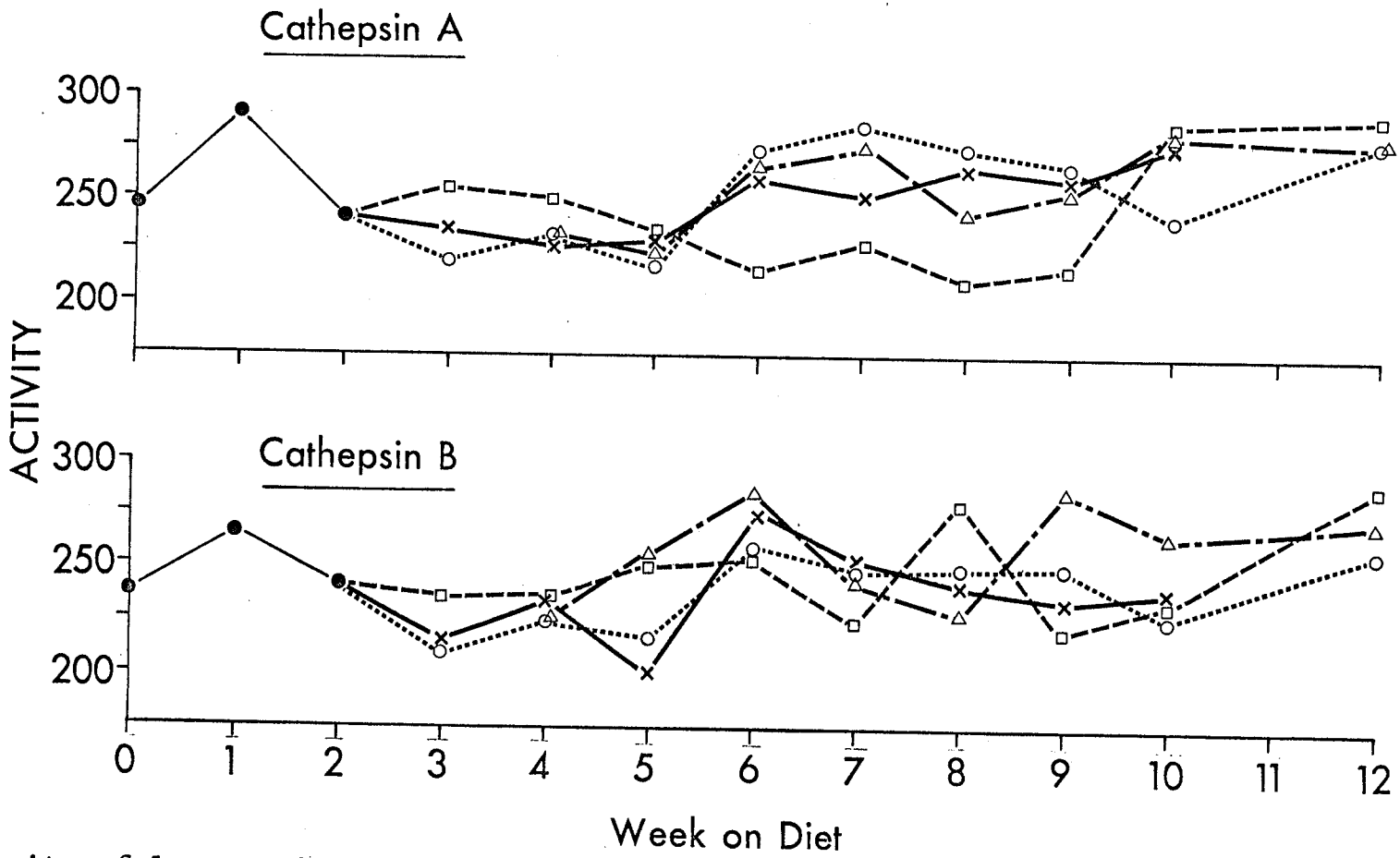

Figure 7. Activity of lysosomal cathepsin A and cathepsin B of guinea pigs fed various amounts of ascorbic acid for 12 weeks. Activity is expressed as units of catheptic activity per milligram protein per 10 minutes. One unit of catheptic activity is expressed as the amount of enzyme which causes an increase of 0.001 units of extinction per 10 minute digestion. The During weeks 0-2 no AA was supplied (●——●) then animals received a stock diet containing 0.5 mg AA/diet (X——X); 10 mg AA/day (□---□); 1 mg (O----O) or 0.5 mg (△--△)/100g body weight/day.

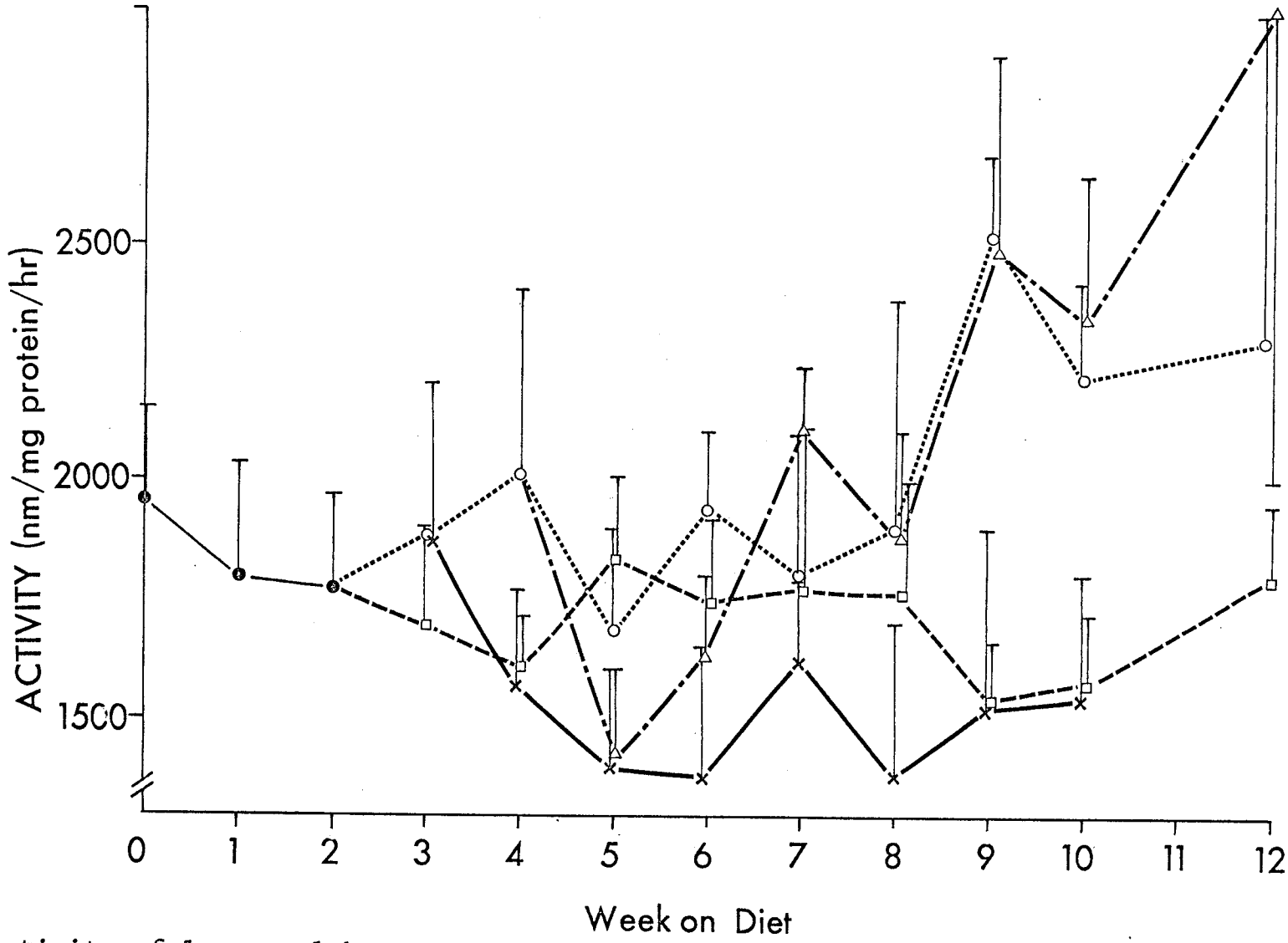

Figure 8. Activity of lysosomal hexosaminidase of guinea pigs receiving various amounts of ascorbic acid for 12 weeks. All values are expressed as nanomoles 4-MU released per milligram protein per hour and represent the mean ± SD. During weeks 0 - 2 no AA was supplied (●—●) then animals received a stock diet containing 0.5 mg AA/day (X——X); 10 mg AA/day (□---□); 1 mg (O----O) or 0.5 mg (△--△)/100g body weight/day.

observed for Groups 1 and 2. In addition, as the experiment proceeded specific activity tended to increase linearly ($P < .01$).

Dietary manipulation also influenced acid phosphatase specific activity (Fig. 9). The pattern of response of Groups 1 and 2 were similar as was the response of Groups 3 and 4. Acid phosphatase increased between week 5 and 10 ($P < .01$, Appendix 4) in those groups receiving the lower concentrations of the vitamin (Groups 2 and 4) but differences were not noted amongst the groups at week 12 (Appendix 4).

5.3.4 Brain catecholamines

AA has been implicated as a cofactor in the synthesis of norepinephrine from dopamine (Levin and Kaufman, 1961). It was considered that if changes in brain norepinephrine and dopamine concentrations were detected, this would serve to demonstrate biochemical deficiency of AA. The effect of the various AA supplements in brain norepinephrine and dopamine is shown in Table 7. Depletion of AA (week 0 - 2) resulted in decreased concentrations (25%) of norepinephrine in the brain. This was accompanied by a corresponding increase (25%) in the concentration of dopamine. When the stock diet of 10 mg AA/day (Groups 1 and 2) were fed, dopamine immediately decreased and norepinephrine increased

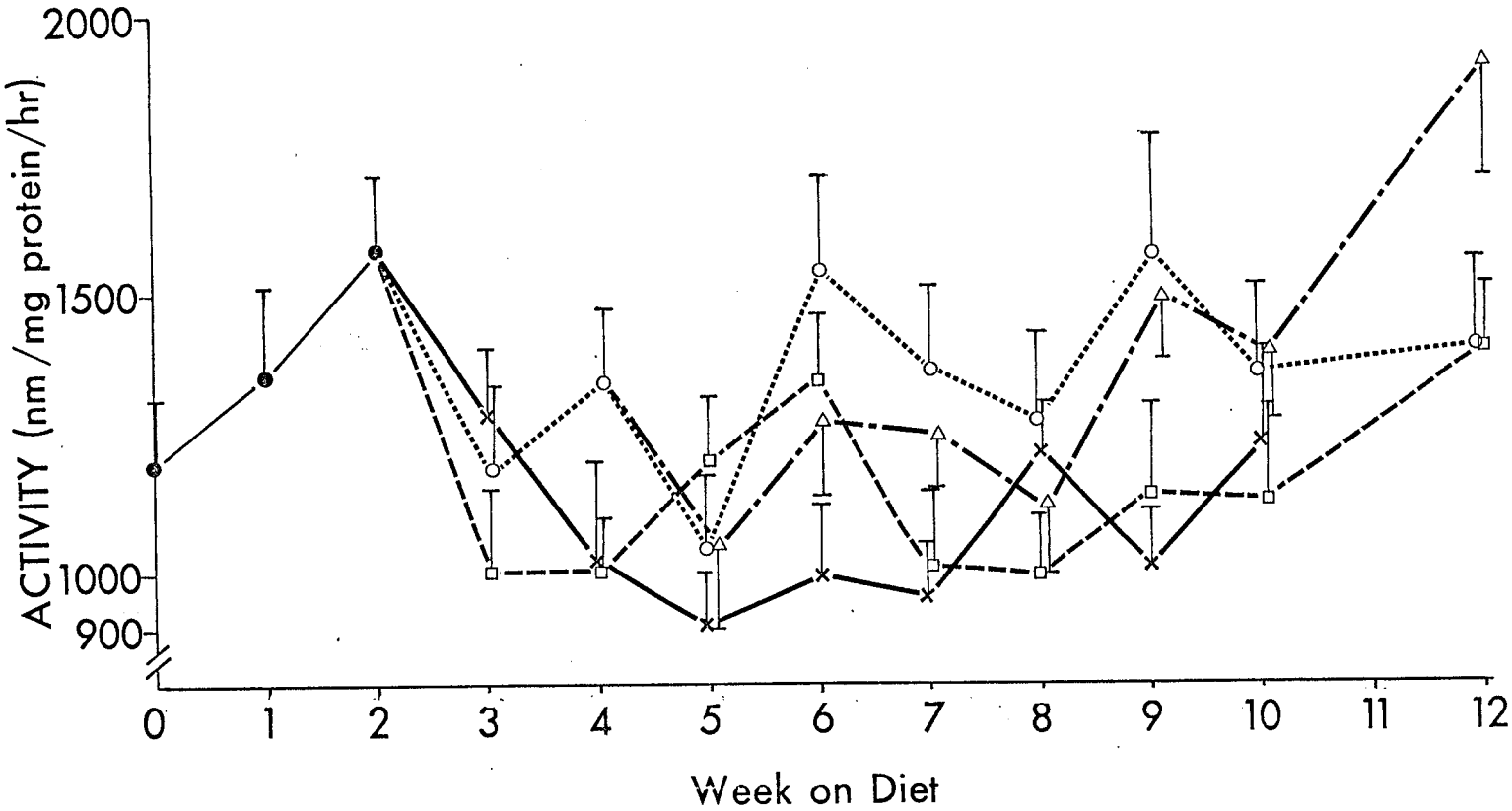

Figure 9. Activity of lysosomal acid phosphatase of guinea pigs fed various amounts of ascorbic acid for 12 weeks. All values are expressed as nanomoles 4-MU released per milligram protein per hour and represent the mean ± SD. The During weeks 0 - 2 no AA was supplied (●——●) then animals received a stock diet containing 0.5 mg AA/day (X——X); 10 mg AA/day (□--□); 1 mg (O---O) or 0.5 mg (△--△)/100g body weight/day.

Table 7

Guinea pig brain norepinephrine and dopamine concentrations[1] when various amounts of ascorbic acid are fed

Protocol	Amine	Week of Experiment 0	1	2					
AA depletion	NE[2]	328+23(4)[3]	282+20(2)	230+16(2)					
	DA[4]	331+6(4)	243+29(2)	403+20(2)					
		Week of Experiment 3	5	6	7	8	9	10	12
AA Supplementation									
0.5 mg AA/g diet	NE	361±19(2)	340±48(2)	367±44(2)	303±35(2)	345±31(2)	354±16(2)	384±25(2)	
	DA	326±11(2)	296±11(2)	324±16(2)	272±13(2)	307±19(2)	371±16(2)	354±15(2)	
10 mg AA/day	NE	404±45(4)	390±14(4)	425±13(4)	321±28(2)	324±13(2)	340±30(2)	322±13(2)	351±13(4)
	DA	374±25(4)	344±16(4)	308±22(4)	283±5(2)	308±22(2)	283±15(2)	288±18(2)	321±21(4)
1 mg AA/100g body wt/day	NE	245±30(4)	265±18(4)	ND	267±8(2)	259±24(2)	291±47(2)	311±46(2)	349±22(4)
	DA	403±19(4)	423±11(4)		399±20(2)	330±21(2)	342±12(2)	356±13(2)	346±21(4)
0.5 mg AA/100g body wt/day	NE		244±18(4)	ND	243±13(2)	ND	251±29(2)	242±20(2)	253±36(4)
	DA		394±13(4)		434±21(2)		400±11(2)	443±30(2)	436±35(4)

1 The numbers represent the mean ± SD and are expressed as ng amine/g wet weight brain.

2 Norepinephrine.

3 Represents number of individual animals.

4 Dopamine.

to normal values. However, with animals receiving 1 mg AA/ 100g body weight per day, this return to normal values was slower and completed by week 8. This return to normal was not observed in those animals receiving 0.5 mg AA a day (Group 4) and the concentration of dopamine and norepinephrine remained significantly different ($P < .05$, Appendix 5 and 6) than values observed at the start of the experiment.

5.4 Discussion

The model for chronic hypovitaminosis C was first documented in 1967 (Ginter et al., 1967) and since then has been used to study the role of AA in cholesterol metabolism (Ginter et al., 1976; Ginter, 1977; Fuminami et al., 1977; Jenkins, 1978). When marginal deficiency of AA is induced in guinea pigs, tissue AA concentrations are substantially decreased while body weight, clinical appearance and food consumption are similar to normal guinea pigs. During the course of this experiment, animals were physically indistinguishable within and from one group to another (Plate 4). This was not unexpected because these animals (Group 4) receiving 0.5 mg AA/100g body weight/day, received the minimum amount of AA for growth (Natl. Acad. Sci. 1972).

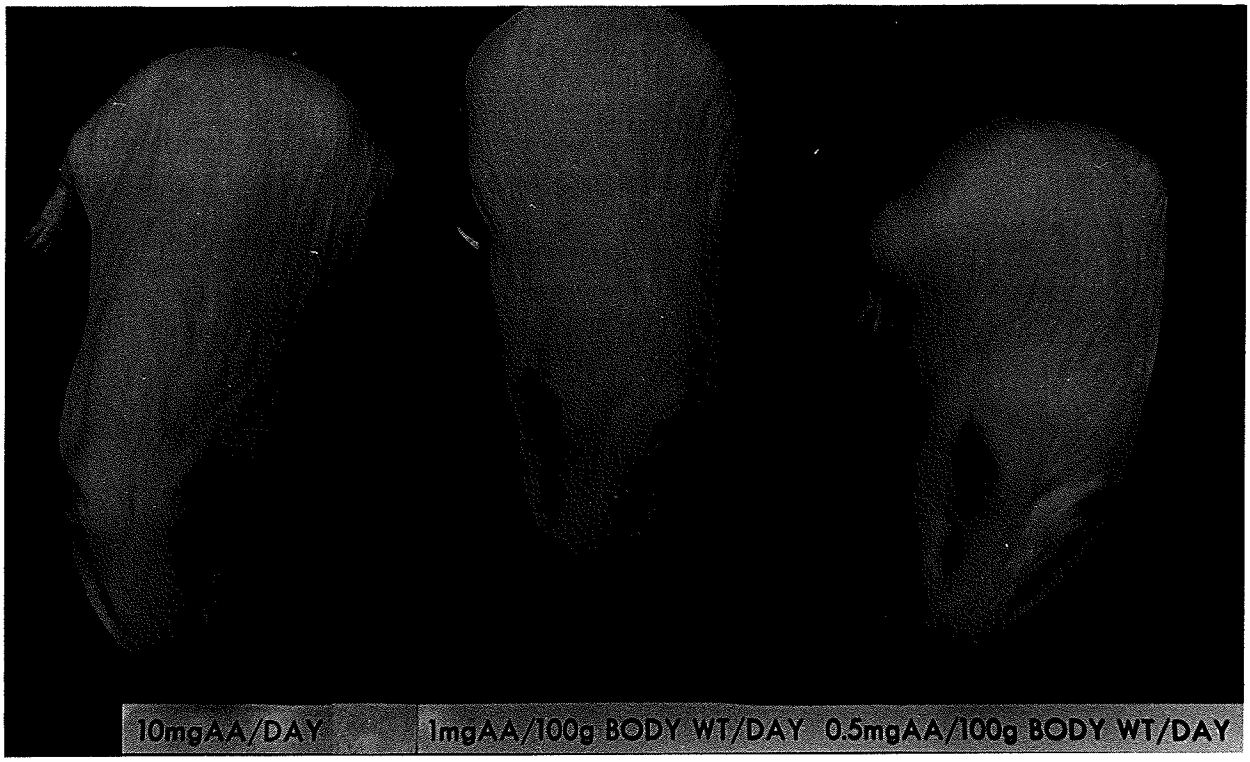

Plate 4: Appearance of guinea pigs fed 10 mg ascorbic acid/day or 1 mg ascorbic acid/100g body weight/day or 0.5 mg ascorbic acid/100g body weight/day for 10 weeks.

Withdrawal of AA from guinea pigs results in rapidly diminishing concentrations of serum and tissue AA (Fig. 5 and 6) (Ginter et al.,1967; 1976; Ginter, 1977). When a small maintenance dose of AA (Groups 3 and 4) is administered, the low concentration of AA in serum and brain persists at approximately the same low values which is similar to that of deficient animals (section 4; Ginter et al.,1967). Administration of higher quantities of AA results in an immediate increase of AA in serum and brain (Fig. 4 and 5). On all regimens, unchanging levels of AA are observed and as the low levels of AA are close to concentrations noted in animals with scurvy (Groups 3 and 4 (section 4) Ginter et al., 1967), these groups may serve as a model for studying the effect of chronic AA depletion. The daily supply of AA prevents the onset of acute deficiency.

When the daily dose of AA is reduced (Group 4) to less than 1 mg/100g body weight, serum and brain AA concentrations decrease (Fig. 4 and 5). The decrease in brain AA is surprising as it is well recognized that this tissue tenaciously conserves AA and responds less readily than serum or liver to AA depletion (Hughes et al.,

1971). This slight, but observable decrease may indicate that other body pools of AA are depleted of AA and that 1 mg AA/100g body weight is sufficient only to maintain those pools at the low AA level acquired during withdrawal of dietary AA (week 0 - 2).

AA has been implicated as a cofactor in several metabolic systems including the synthesis of norepinephrine, a neurotransmitter in the central nervous system (Levin and Kaufman, 1961). Changes in AA levels of brain tissue are accompanied by changes in brain norepinephrine and dopamine concentrations (Deana et al., 1975; Izquierdo et al., 1968). Thus, if differences in the concentrations of these two amines were noted, this would suggest that a biochemical deficiency of AA exists. Table 7 illustrates that, brain norepinephrine and dopamine levels change in response to brain AA depletion, and on repletion of AA catecholamine values return to within normal ranges (Groups 1 and 2). However, brain AA remains at a steady low state in those animals receiving 1 mg or 0.5 mg AA/100g body weight per day (Fig. 5). By week 8 brain norepinephrine and dopamine have returned to normal, in Group 3, illustrating that some adaption to the low supply of AA has taken place, but is

only accomplished slowly. Furthermore, this return to pre-experimental values was not observed for Group 4, hence this group of animals can be taken as biochemically deficient in AA.

In view of the report by Mell and Gustafson (1977) that AA interferes with the trihydroxyindole technique used for measuring norepinephrine and gives falsely high values, it was important to ascertain that the observations in Table 7 were not artifactual. Brain tissue obtained from Groups 3 and 4 was supplemented with AA equivalent to that amount present in brain tissue found in Groups 1 and 2. Regardless of whether the tissue was supplemented or not, similar levels of the amines were obtained. The trihydroxyindole procedure does not interfere with the analysis of dopamine. An explanation for the increase of dopamine in response to diminished concentrations of AA is that the enzyme, dopamine β-hydroxylase, responsible for hydroxylating dopamine to norepinephrine, is not functioning as efficiently because of decreased availability of AA. Deana et al. (1975) noted that norepinephrine and dopamine values differed as much as 50% from normal in acutely deficient animals. This response is similar to our findings,

although the magnitude of the response noted by Deana et al. was greater. Greene and coworkers (1979) also examined the effect of acute AA deficiency on brain dopamine and norepinephrine. These authors showed no measurable differences from normal values in brain norepinephrine and dopamine.

Previous results showed that the lysosomal enzymes of the liver were more sensitive than the brain enzymes to changes in AA concentration, (section 4) suggesting that apparent preferential changes of hepatic lysosomal hydrolases may depend on specific metabolic requirements of the organ. Furthermore, not all enzymes were affected in acutely deficient animals. For example, hexosaminidase, acid phosphatase, α-galactosidase and α-mannosidase were elevated 2 - 3 fold higher than normal, whereas there was no change in the specific activities of arylsulphatase A and B, acid lipase, β-glucosidase and cathepsins, A, B, D and E (Table 5). Similar observations are noted in the present experiment. Lysosomal cathepsins A and B (Fig. 7) were unaffected during the course of the experiment, whereas the specific activities of hexosaminidase (Fig. 8) and acid phosphatase (Fig. 9) changed. The response of these two enzymes to dietary AA differed, the specific activity of

hexosaminidase continuously increased over the experimental period in groups 3 and 4, but was not observed for acid phosphatase. The specific activity of acid phosphatase was elevated by week 6 (Groups 3 and 4) and no further increase was noted during the course of the experiment. There are several reports in the literature that distinct populations of lysosomes can be identified from spleen and thymus (Bowers and deDuve, 1967), retina (Sialkotos et al., 1978), liver (Stahn et al., 1970), leucocytes (Rest et al., 1978) and fibroblasts (Rome et al., 1979). In the present experiment, the changes observed in the specific activities of lysosomal hexosaminidase and acid phosphatase but not with cathepsins A and B, and may reflect changes in specific populations of lysosomes. Consequently, lysosomes may have different enzyme complements which are affected to different degrees by factors such as dietary stress and the effects observed with marginally AA deficient guinea pigs may simply be the result of changes in specific populations of lysosomes. Alternatively, the changes in lysosomal hexosaminidase and acid phosphatase may be the consequence of increased synthesis of these proteins, decreased degradation of enzymic proteins or a combination of these. In this experiment, these alternatives were not determined.

6. IN VITRO STUDIES ON THE INHIBITION OF HEXOSAMINIDASE BY ASCORBIC ACID

6.1 Introduction

AA was shown to inhibit hexosaminidase of guinea pig liver homogenates (Table 3) as well as the partially purified enzyme prepared from bovine serum albumin (Kanfer and Spielvogel, 1973). β-glucuronidase of lysosomal suspensions was also found to be inhibited by AA (Dolbeare and Martlage, 1972). In vitro AA has the capacity of cleaving covalent bonds (Robinson et al., 1973; Maejima and Kresheckx, 1971; Westhall et al., 1976; Kanfer and Spielvogel, 1976). In vivo studies demonstrated that the specific activities of various lysosomal hydrolases are elevated in acutely AA deficient guinea pigs (Table 5). In chronically AA deficient guinea pigs, showing no overt clinical signs of AA deficiency, the specific activity of hexosaminidase (Fig. 8) and acid phosphatase (Fig. 9) were significantly higher as compared to control animals, whereas the specific activity of cathepsins A and B remained constant (Fig. 7). Thus, it was of interest to examine the properties of AA inhibition of lysosomal enzymes from normal control, scorbutic and starved control animals to

investigate the possibility that AA may be a small regulatory molecule. Lysosomal hexosaminidase was chosen for these studies as the characteristics of the purified enzyme have been documented (Geiger and Arnon, 1976; Lee and Yoshida, 1976; Freeze et al., 1979).

6.2 Experimental procedures

Normal control scorbutic and starved control guinea pigs were maintained as described in section 5.2.1. Lysosomes were isolated from individual livers of 16 normal or 11 scorbutic or 6 starved control guinea pigs as previously described (section 3.2.2). The lysosomal pellets obtained were suspended in distilled deionized water and then pooled on an equal protein basis and stored frozen at -20^{o}C until required for analysis. A sample was not used more than 5 times.

AA, total lysosomal hexosaminidase and hexosaminidase A were determined (sections 3.3.4., 3.3.1). The solutions of AA and AA analogues were adjusted to pH 4.5 with 2.5 N KOH prior to being added to the incubation mixture. Km and Ki values were obtained by the double reciprocal plot procedure of Lineweaver and Burke (1934). All values were adjusted

for non-enzymatic cleavage of the substrate by AA and the effects of the various chemicals on enzymatic activity and on the interference of the fluorescence measurement were assessed and the experimental values corrected.

6.3 Results

6.3.1 Inhibition of hexosaminidase by ascorbic acid

The ability of AA to inhibit the hydrolysis of 4MU-GlcNac by lysosomes from normal control, scorbutic and starved control guinea pigs is shown in Fig. 10. In these studies, 8 µg of lysosomal protein were used with varying amounts of AA (0-100 µmoles, 0-500 mM). It is apparent that large amounts of the vitamin are necessary for hexosaminidase inhibition. In the presence of 100 µmoles (500 mM) of AA, no more than 70% inhibition is observed. Furthermore, the amount of inhibition observed among the three groups is similar. Inhibition per se was not due to any changes in the pH of the incubation medium and there was no change in the shapes of the curves obtained as a function of pH by the addition of AA (2.5, 5.0 and 50.0 mM; Fig. 11). The Lineweaver-Burke plots of hexosaminidase activity in the presence of 10 or 50 µmoles AA (50 or 250 mM) of normal control, scorbutic and starved control

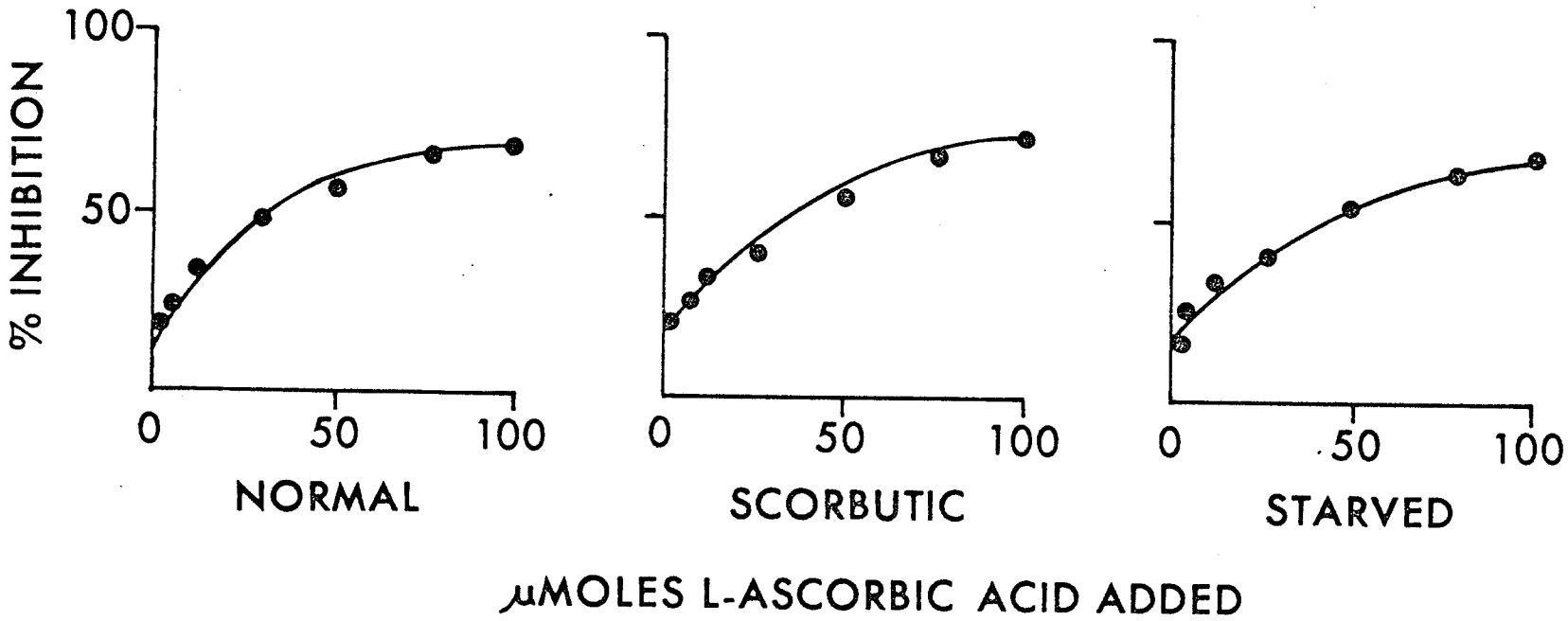

Figure 10. Inhibition of 4MU-GlcNac hydrolytic activity of lysosomes from normal control, scorbutic or starved control guinea pig liver by L-ascorbic acid. The total volume of the reaction mixture was 0.2 ml.

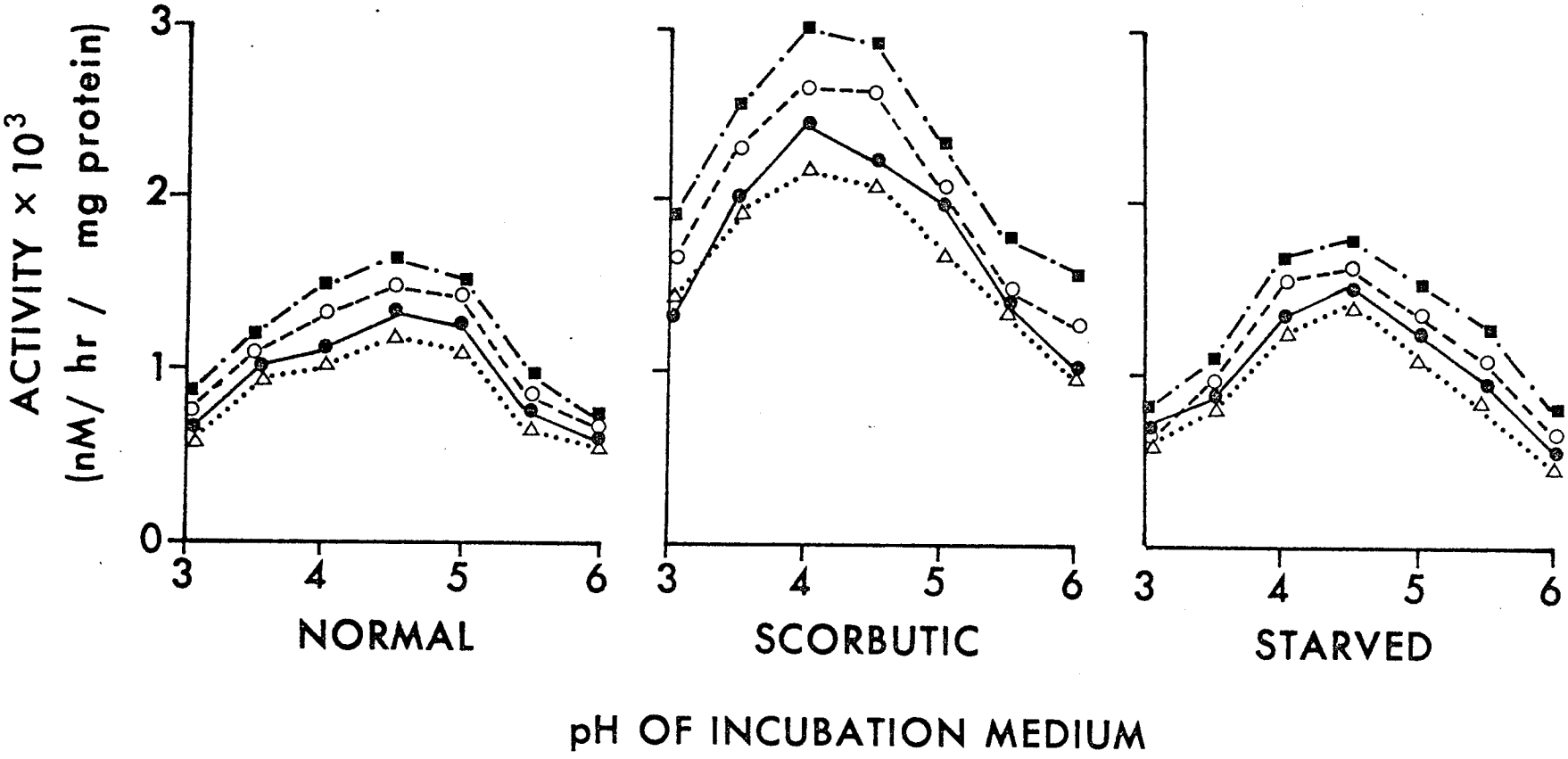

Figure 11. The effect of pH and varying amounts of L-ascorbic acid on the activity of hexosaminidase from normal control, scorbutic and starved control guinea pigs. The lines represent activity in the absence (■-·-·-■) and presence of 2.5 mM (O---O), 5.0 mM (●——●) and 50 mM (△·····△) ascorbic acid.

guinea pig lysosomes (Fig. 12, 13 and 14) illustrates that the inhibition was non competitive. The Km and Ki were 5.2×10^{-4} M and 0.18 M, respectively.

Hexosaminidase can be separated by electrophoretic or column chromatographic procedures into two predominant isoenzyme forms, hexosaminidase 'A' and hexosaminidase 'B' (Robinson and Stirling, 1968). Since mg amounts of the vitamin to μg of lysosomal protein were required to inhibit hexosaminidase, it was of interest to see if both isoenzymes were inhibited to a similar extent among the three groups. Table 8 shows that regardless of the group, both hexosaminidase A and B are inhibited similarly as the relative proportions of these isoenzymes remain unchanged.

The inhibition was reversed by separating the lysosomal protein from AA by gel filtration as shown in Table 9. Most of the enzyme activity appeared in fraction 2 and the bulk of the AA was found to be in fraction 3. 97% or 96% or 94% of the original enzyme activity and 77% or 85% or 81% of the AA were recovered from normal control, scorbutic or starved control guinea pigs.

6.3.2 Specificity of hexosaminidase inhibition

L-Ascorbic acid has vitamin C activity, D-ascorbate

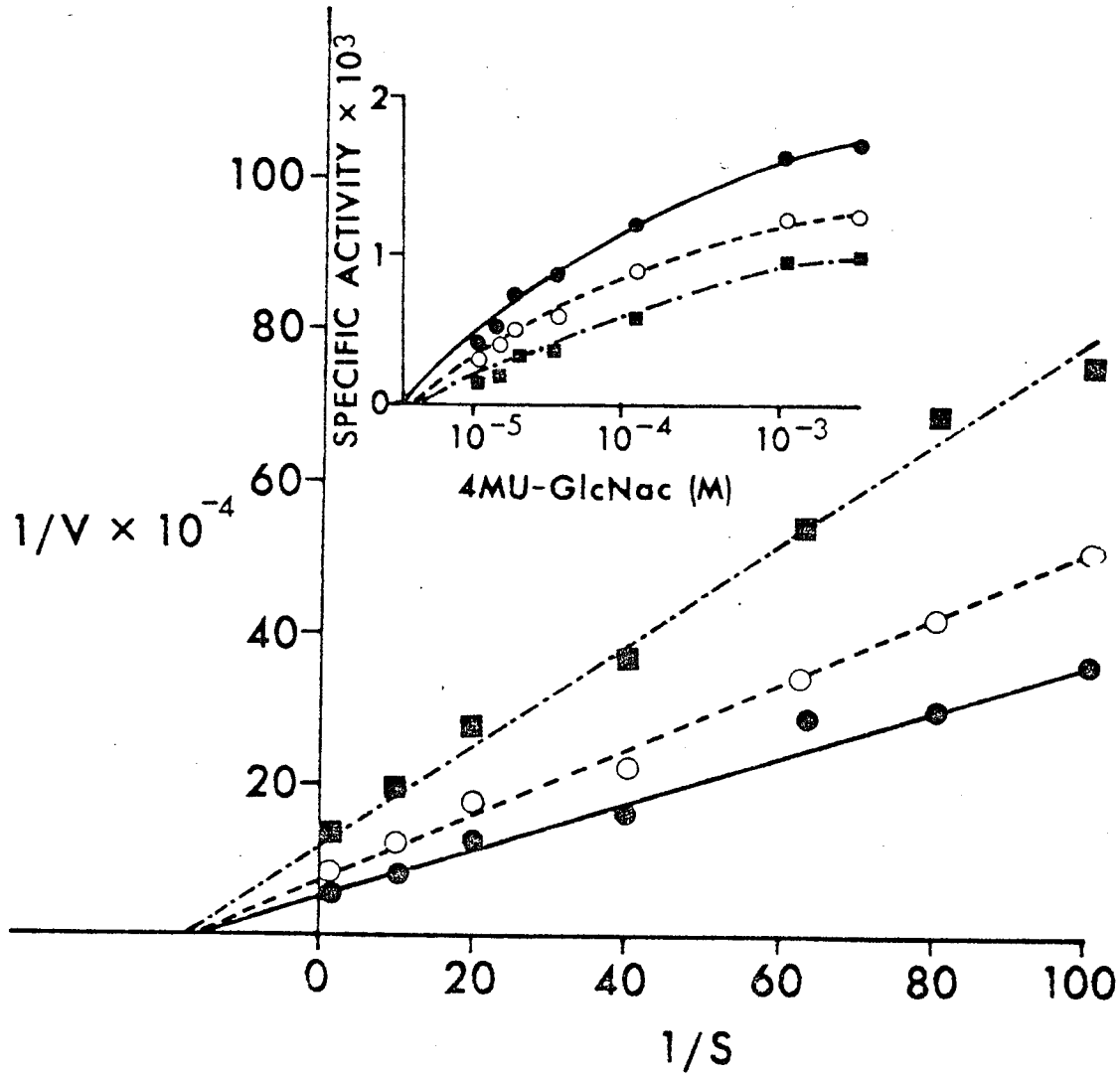

Figure 12. Lineweaver-Burke plot of the effect of varying concentrations of 4MU-GlcNac upon the rate of hydrolysis of normal control guinea pig lysosomes in the absence (●——●) and presence of 10 (O·····O) and 50 (■—·—■) μmoles of L-ascorbic acid. The total volume of the reaction mixture was 0.2 ml.

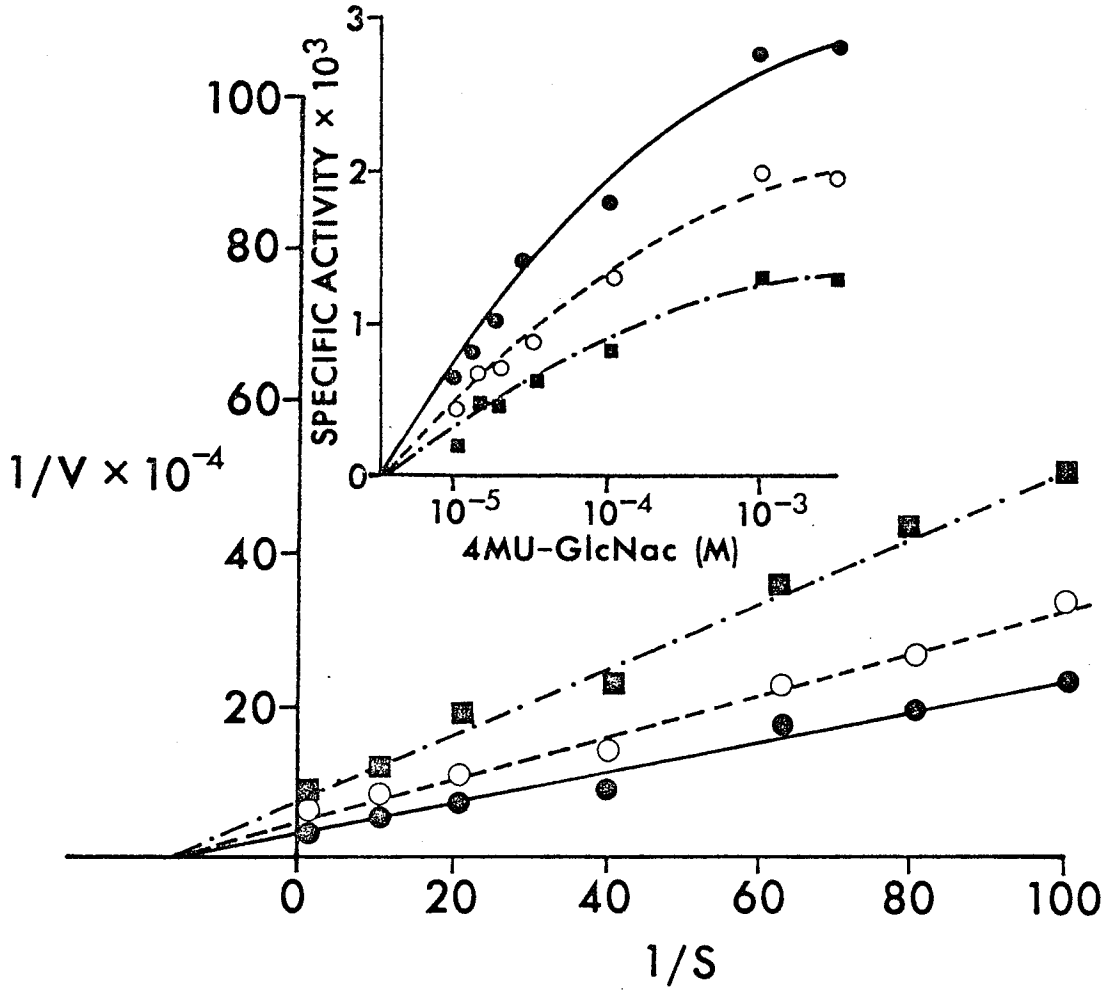

Figure 13. Lineweaver-Burke plot of the effect of varying concentrations of 4MU-GlcNac upon the rate of hydrolysis of scorbutic guinea pig lysosomes in the absence (●——●) and presence of 10 (O---O) and 50 (■-·-·-■) μmoles of L-ascorbic acid. The total volume of the reaction mixture was 0.2 ml.

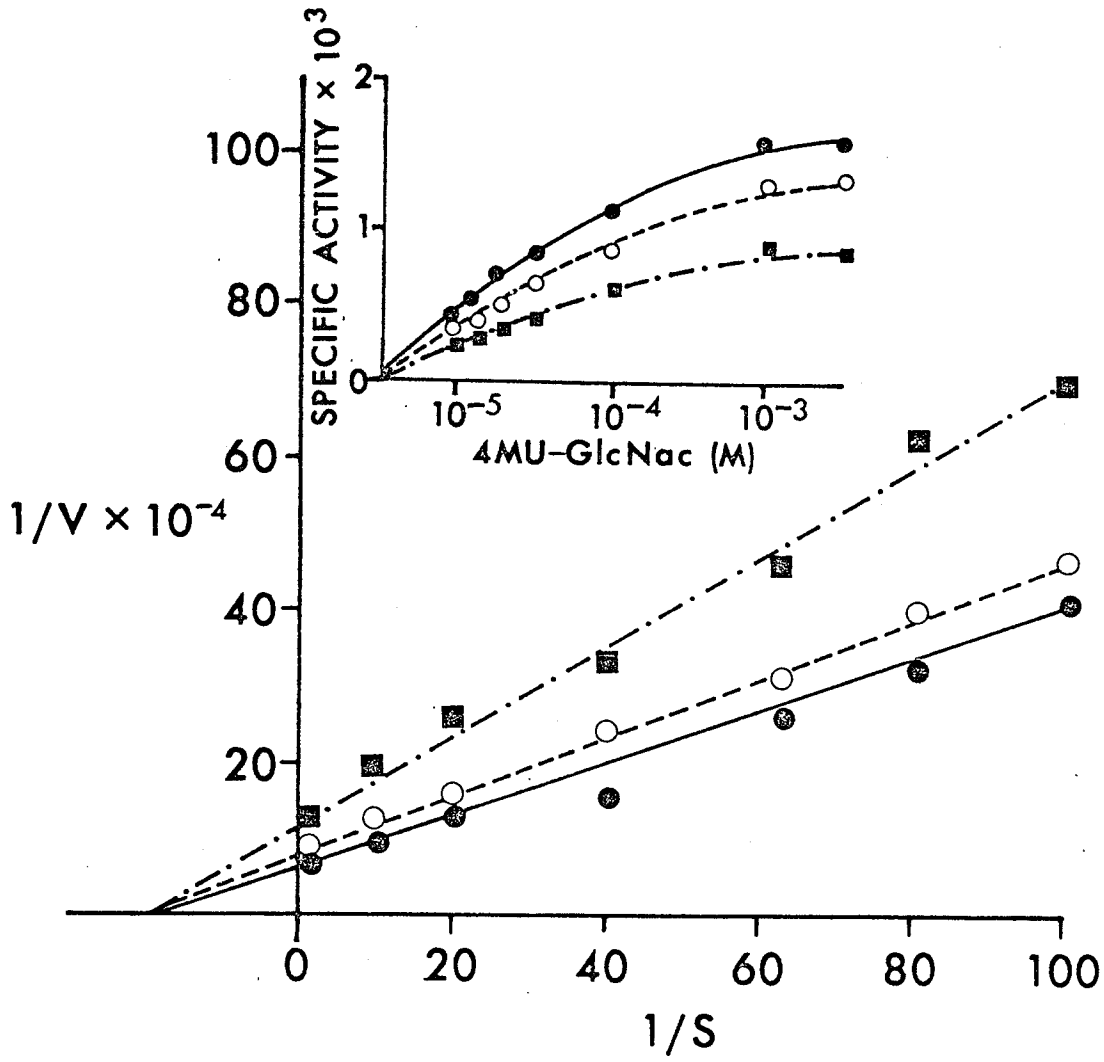

Figure 14. Lineweaver-Burke plot of varying concentrations of 4MU-GlcNac upon the rate of hydrolysis of starved control guinea pig lysosomes in the absence (●——●) and presence of 10 (O- - -O) and 50 (■-·-·-■) μmoles of L-ascorbic acid. The total volume of the reaction mixture was 0.2 ml.

Table 8

The effect of L-ascorbic acid on lysosomal hexosaminidase isoenzymes A and B from normal control, scorbutic and starved control guinea pigs

Group	Ascorbic acid concentration	Activity[1] of hexosaminidase A	B
Normal	0	967(60)[2]	630(40)
	50 mM	632(61)	408(39)
	% inhibition	35	35
Scorbutic	0	1793(60)	1196(40)
	50 mM	1240(61)	801(39)
	% inhibition	31	33
Starved	0	935(59)	645(41)
	50 mM	511(60)	340(40)
	% inhibition	34	36

[1] Activity is expressed as nanomoles 4MU released/hour/mg protein.

[2] Values in parentheses indicate % of total detectable hexosaminidase as A and B components.

Table 9

Reversibility of L-ascorbic acid inhibition of lysosomal hexosaminidase activity[1] by G200 gel filtration[2] from normal controls, scorbutic and starved control animals

Group		Recovery of Hexosaminidase activity	Recovery of Ascorbic Acid (μg)
Normal	Fraction 1	1.9	28.5
	Fraction 2	11.2	75.3
	Fraction 3	0.1	1255.0
	% Recovery	97.0	77.0
Scorbutic	Fraction 1	1.9	30.0
	Fraction 2	15.6	87.8
	Fraction 3	0.6	1384.7
	% Recovery	96.0	85.0
Starved	Fraction 1	2.1	29.3
	Fraction 2	9.2	83.4
	Fraction 3	0.4	1310.1
	% Recovery	94.0	81.0

[1] Activity is expressed as nanomoles 4MU released/hr/8.2, 6.1 or 7.5 μg protein from normal, scorbutic and starved guinea pig lysosomes. Routinely, 8.2 μg or 6.1 μg or 7.5 μg of lysosomal protein yielded 13.7, 18.8 or 12.4 nanomoles 4 MU in the absence of AA, whereas in the presence of 10 μmoles AA the activity was decreased by 36 or 42 or 37% to 8.8 or 10.9 or 7.8 nmoles 4 MU released.

[2] Lysosomal protein and AA were incubated in .25 M citrate-phosphate buffer, pH 4.5 (200 μl total volume) at 37° for 30 minutes. The buffer-protein-AA mixture was applied to a 1 x 0.4 cm Sephadex G200 column (Pharmacia Fine Chemicals Inc., Piscataway, N.J.) equilibrated with 0.25 M citrate-phosphate buffer, pH 4.5. The enzyme activity and AA were eluted from the column with the same buffer and 3 fractions of 1 ml each (fraction 1, 2 and 3) were collected. Each fraction was assayed for hexosaminidase activity and AA as described under materials and methods.

and D-araboascorbate have little vitamin C potency (Burns et al.,1957; Hughes and Jones, 1970), whereas glucoascorbic acid has no vitamin C activity (Reichstein et al., 1934). Thus, the specificity of AA inhibition was examined. Regardless of the group, a similar degree of inhibition was observed with D-ascorbate and D-araboascorbate, although the magnitude of the inhibition was less than with L-AA (Table 10). Glucoascorbic acid and dehydroascorbate were without effect.

As AA is a weak acid (pK 4.17), the effects of other organic acids (2.5 - 50.0 mM) with pKs similar to that of AA were examined. Benzoic acid (pK 4.19), succinic acid (pK 4.16) and oxalic acid (pK 4.19) had little effect on hexosaminidase activity (Table 11) although 50.0 mM succinic acid inhibited hexosaminidase to an extent similar to that observed with 50 mM AA (Table 10).

The effects of various reducing agents on hexosaminidase activity from normal control, scorbutic and starved control guinea pig lysosomes are shown in Table 12; glutathione, cysteine, β-mercaptoethanol and NADH were without effect.

6.3.3 Effects of O_2 and metal chelators on hexosaminidase activity

Table 10

Inhibition by ascorbic acid and ascorbate analogues of lysosomal hexosaminidase activity from normal control, scorbutic and starved control guinea pigs

Group	Addition	mM final concentration		
		2.5	5.0	50.0
Normal	L-ascorbic acid[1]	11	20	28
	D-ascorbic acid	9	11	15
	D-araboascorbic acid[2]	16	17	20
	D-glucoascorbic acid	2	0	2
	Dehydroascorbic acid[3]	2	2	
Scorbutic	L-ascorbic acid	10	23	30
	D-ascorbic acid	6	9	15
	D-araboascorbic acid[2]	16	16	17
	D-glucoascorbic acid	0	1	0
	Dehydroascorbic acid[3]	2	2	
Starved	L-ascorbic acid	9	21	29
	D-ascorbic acid	8	12	16
	D-araboascorbic acid[2]	10	14	19
	D-glucoascorbic acid	1	1	2
	Dehydroascorbic acid[3]	0	1	

[1] The numbers represent the percent inhibition observed in the presence of each addition to the incubation mixture.

[2] The concentrations (mM) of D-araboascorbic acid used were 2.5, 5.0 and 10.0.

[3] The concentrations (mM) of dehydroascorbic acid used were 1.5 and 3.0.

Table 11

The effects of various organic acids on lysosomal hexosaminidase activity[1] from normal control, scorbutic and starved control guinea pigs

Group	Concentration	Benzoic Acid	Succinic Acid	Oxalic Acid
Normal	0	1628	1628	1628
	2.5 mM	1646	1594	1702
	5.0 mM	1609	1589	1693
	50.0 mM	1701[2]	1254	1714
Scorbutic	0	2994	2994	2994
	2.5 mM	2918	2728	2974
	5.0 mM	2904	2784	2760
	50.0 mM	2821[2]	2089	2961
Starved	0	1695	1695	1695
	2.5 mM	1577	1627	1608
	5.0 mM	1653	1647	1666
	50.0 mM	1620[2]	1157	1636

[1] Activity is expressed as nanomoles 4 MU released/mg protein/hour.

[2] 10 mM.

Table 12

The effects of various reducing agents on lysosomal hexosaminidase activity[1] from normal control, scorbutic and starved control guinea pigs

Group	Concentration	Cysteine	Glutathione	β-mercaptoethanol	$NADH_2$
Normal	0	1621	1621	1621	1689[5]
	2.5 mM	1625	1599	1596[2]	1681[6]
	5.0 mM	1666	1591	1591[3]	1658[7]
	10.0 mM	1656	1576	1571[4]	1707
Scorbutic	0	3054	3054	3054	3054[5]
	2.5 mM	3173	3173	2996[2]	3020[6]
	5.0 mM	3097	3097	2971[3]	2990[7]
	10.0 mM	3173	3016	2872[4]	2944
Starved	0	1602	1602	1602	1602[5]
	2.5 mM	1581	1663	1587[2]	1684[6]
	5.0 mM	1626	1626	1599[3]	1675[7]
	10.0 mM	1592	1605	1535[4]	1688

[1] Activity is expressed as nanomoles 4 MU released/hour/mg protein.
[2] 3.75 mM
[3] 7.5 mM
[4] 11.0 mM
[5] 150 mM
[6] 300 mM
[7] 450 mM

In reactions involving AA, such as the hydroxylation of proline, molecular oxygen and ferrous ions are required (Barnes and Kodicek, 1972). Anaerobic incubation of lysosomal protein with AA prevented the inhibition of the enzyme activity in lysosomes from each of the 3 groups, as did the addition (up to 10 mM) EDTA, EGTA, salicylic acid and o-phenanthroline (Table 13). In the absence of AA, these chelators did not affect hexosaminidase activity.

6.3.4 Effects of free radical scavengers on hexosaminidase activity

As AA inhibition of hexosaminidase is observed under aerobic conditions, free radicals such as the superoxide anion, singlet oxygen and hydroxyl radicals as well as hydrogen peroxide may be generated which could be responsible for hexosaminidase inhibition. AA (10 µmoles) inhibition of hexosaminidase from normal control, scorbutic or starved control guinea pig lysosomes was not prevented by the addition of 50 mM diethylsulfide (dissolved in 10 mM ethanol) or 50 mM hydroquinone, known scavengers of singlet oxygen (Foote et al.,1970); 10 mM Tiron or 100 µg superoxide dismutase, known scavengers of superoxide anions (Paine, 1978); nor by scavengers of hydroxyl radicals such as

Table 13

The effects of various chelating agents on L-ascorbic acid inhibition of lysosomal hexosaminidase activity[1] from normal control, scorbutic and starved control guinea pigs

Group	mM chelator	0.1	0.5	1.0	5.0	10.0
Normal	EDTA	1268	1363	1238	1328	1459
	EGTA	1504	1498	1463	1557	1484
	Salicylic acid	1422	1471	1602	1527	1451
	o-Phenanthroline	1481	1521	1456	1552	1464
Scorbutic	EDTA	2471	2494	2644	2699	2835
	EGTA	2538	2755	2897	2813	2736
	Salicylic acid	2631	2661	2684	2649	2596
	o-Phenanthroline	2766	2755	2528	2673	2718
Starved	EDTA	1419	1417	1568	1469	1575
	EGTA	1509	1719	1597	1508	1686
	Salicylic acid	1480	1497	1552	1729	1591
	o-Phenanthroline	1409	1713	1659	1646	1583

[1] Activity is expressed as nanomoles 4MU released/hour/mg protein. The activity of hexosaminidase in the absence and presence of 2.5 mM ascorbic acid was 1560 and 1377 for normal control, 2812 and 2418 for scorbutic and 1544 and 1351 for starved control guinea pigs.

sodium benzoate (20 mM) or ethanol (50 mM) (Paine, 1978). Catalase and peroxidase scavenge hydrogen peroxide. The addition of 0.5 units of catalase or peroxidase effectively abolished AA (10 µmoles) inhibition of hexosaminidase. However, no inhibition of the enzyme was observed in the presence of 50 mM hydrogen peroxide.

6.4 Discussion

It is evident from the data obtained on AA inhibition of hexosaminidase that no discernable differences exist for each of the parameters examined among lysosomes isolated from normal control, scorbutic or starved control guinea pigs. Kanfer and Spielvogel (1973) demonstrated the inhibition of hexosaminidase by various sugar lactones, including AA. These authors indicated that the sole common feature of these compounds was the lactone ring and that the sugar acids were not inhibitory. Glucoascorbic acid, a 7 carbon sugar structurally similar to AA, had no inhibitory properties, thus the presence of the lactone ring is not the only prerequisite for inhibition. AA inhibition of hexosaminidase from normal control, scorbutic and starved control guinea pig lysosomes was

noncompetitive (Fig. 12, 13 and 14) indicating that the inhibitor binds to some site other than the active site on the enzyme. This is in contrast to that previously observed by Kanfer and Spielvogel (1973) with rat liver lysosomes and the partially purified enzyme obtained from bovine serum albumin. These authors demonstrated that inhibition was of the 'mixed' type as both the Km and the Vmax were changed.

In view of the high concentration of AA required for inhibition, 50% inhibition required 200 mM AA per 8 μg lysosomal protein, the inhibitory properties of AA may be of little physiological importance. Isolated lysosomes were analyzed for AA, but none was found associated with these particles, all the AA was found in the supernatant.

The involvement of AA has been demonstrated in the hydroxylation of proline (Barnes and Kodicek, 1972) and dopamine (Levin et al., 1960; 1961) as well as in tyrosine catabolism (Zannoni and LaDu, 1960; 1962). These studies indicated that AA is not specifically required in vitro, as other reducing agents such as D-ascorbate, D-araboascorbate, SH-compounds, reduced pteridines and the dye 2, 6-dichloroindophenol could replace AA. Reducing agents (Table 12) and organic acids with pKs similar to

AA, with the exception of 50 mM succinate, (Table 11) were not inhibitory for hexosaminidase. 50 mM Succinate and 50 mM AA inhibited hexosaminidase to a similar extent (Table 10). Dehydroascorbate and glucoascorbate (Table 10) had little effect on hexosaminidase activity, although D-ascorbic acid and araboascorbic acid, compounds with little vitamin C potency (Burns et al., 1957; Hughes and Jones, 1970) slightly inhibited the enzyme.

AA is capable of oxidation, first to an unstable free radical, monodehydroascorbic acid, and then to the more fully oxidized dehydroascorbic acid. This reaction is mediated in vitro by catalytic amounts of metal (Dawson and Tokrayama, 1961). The inhibition of hexosaminidase required the presence of oxygen and metals since anaerobic conditions and metal chelators (Table 13) prevented the inhibition, suggesting that AA per se was not the inhibiting agent. Furthermore, specific scavengers of superoxide anions, singlet oxygen and hydroxyl radicals did not affect AA inhibition of hexosaminidase, thus it is inferred that these radicals had no effect on hexosaminidase inhibition. The addition of catalase and peroxidase, abolished the inhibition, but it is doubtful that hydrogen peroxide is

the inhibitory molecule as this compound (50 mM) had no effect on hexosaminidase activity. AA has been shown to accelerate the formation of catalase and peroxidase compound II, and to stabilize this compound once it is formed (Keilin and Nicholls, 1958). AA is not oxidized in the coupled peroxidatic reaction catalyzed by catalase, but is easily oxidized to dehydroascorbate by peroxidase (Keilin and Nicholls, 1958). Consequently, as dehydroascorbic acid was not inhibitory (Table 10), and as AA inhibition required oxygen and a metal, whereas no active oxygen radical appears to be involved, it is assumed that monodehydroascorbate or some intermediate in the oxidation of AA to dehydroascorbic acid is the species responsible for hexosaminidase inhibition. Whether the generation of this species is a prerequisite for binding to the enzyme, or whether AA binds and then the reactive species generated, has not been determined.

Although many studies have been carried out on the properties of mammalian hexosaminidase, there is still much to know of the regulation of this enzyme. AA was shown to inhibit hexosaminidase. However, the high vitamin concentrations necessary for inhibition and the similarities

of AA inhibition of hexosaminidase of lysosomes obtained from normal control, scorbutic and starved control guinea pigs, detracts from any physiological role the vitamin may have in the regulation of this enzyme.

7. CONCLUSION

The hypothesis that L-ascorbic acid may be a physiological regulator of lysosomal enzyme activity was investigated. In vivo studies using guinea pigs acutely and chronically deficient in the vitamin as well as in vitro studies were used to examine the hypothesis. The observations from these studies are discussed in the following section.

Lysosomal enzymes play important roles in degrading the majority of intracellular and extracellular macromolecules, the products of digestion being used for energy production and precursors for essential tissue reconstitution. In this way lysosomal acid hydrolases are important in conditions where dietary nutrients are unavailable. In the livers of scorbutic guinea pigs less protein was found (Table 6) which may partly reflect inadequate food intake as diminished protein concentration was also observed in starved control animals. This diminished protein concentration may partly account for the increases in activities of acid hydrolases in scorbutic and starved control guinea pigs as the total activity of hexosaminidase was similar in these groups. However,

when compared to normal control animals, the total activity of hexosaminidase present in scorbutic and starved control animals was higher indicating the presence of more enzyme protein or increased enzymatic activity.

Terroine and Hitier (1969) Hitier (1968) and Terroine (1971) noted decreased protein concentration and increased total activity of RNase, DNase and acid phosphatase in skeletal muscle and testicular tissue of scorbutic guinea pigs when compared to control animals. These authors ascribed the increase in activity to more lysosomal enzymes rather than increased enzymatic activity as electron micrographs showed increased numbers of secondary lysosomes. The presence of dense bodies in electron micrographs is usually ascribed to lysosomes but in the present study with scorbutic guinea pigs, electron micrographs did not show any increased numbers of secondary lysosomes (Plates 1, 2 and 3; Appendix 7). However, lysosomes do not always appear as dense bodies in electron micrographs (Hers and Van Hoof, 1973) and morphological studies should be used with stains for acid phosphatase to unequivocally demonstrate the presence of lysosomes. (Acid phosphatase staining was not undertaken

by Dr. Hoogstraten as his laboratory was not equipped for acid phosphatase staining.) Thus, there may be an increased population of secondary lysosomes not identified in the electron micrographs of hepatocytes of scorbutic animals. Moreover, the increased recovery of lysosomal protein from scorbutic animals suggested the presence of a greater number or the presence of enlarged lysosomes (Table 6).

Studies with tissue homogenates require cautious interpretation because of the possibility that hydrolytic enzymes with acid pH optima may be located at cellular sites other than in lysosomes (Neil and Horner, 1964; Fishman et al., 1967; Marsh and Goulay, 1971). Isolation of lysosomes from liver of scorbutic guinea pigs and control groups, showed increased specific activities of some lysosomal enzymes only in lysosomes isolated from scorbutic animals (Table 5). Not all of the lysosomal enzymes examined were elevated and those enzymes that were increased were elevated to different degrees. This may reflect the heterogeneity of lysosomal enzymes in the liver rather than any specific effect of AA on lysosomal enzymes.

It is difficult to ascribe the changes observed in

enzyme activities solely to a lack of AA. Scurvy is a complicated state characterized by rapid weight loss, negative nitrogen balance, impaired tricarboxylic acid cycle, subcutaneous haemorrhages, impaired collagen synthesis, imminent death as well as low tissue AA concentrations. Any or a combination of these effects could increase the activity of lysosomal enzymes. For example, Desai (1969) reported increased activities of acid phosphatase, β-glucuronidase, α-galactosidase, aryl sulphatase and cathepsin in the starving rat. Assuming that in the study with scorbutic guinea pigs the assimilation and utilization of food was similar among the groups, starvation alone cannot account for the increased specific activities observed for hexosaminidase, acid phosphatase, α-galactosidase, α-mannosidase and β-glucuronidase in scorbutic animals (Table 5). Although the weights of scorbutic and pair fed animals were similar (Fig. 1), specific activities of enzymes in lysosomes isolated from starved control animals were similar to those of animals not subjected to any dietary stress (Table 5).

Various vitamin deficiencies and excesses increase the activities of lysosomal enzymes. In conditions of

both vitamin A excess and deficiency, acid phosphatase and cathepsin activities increased in rat liver (Dingle et al., 1969), whereas in vitamin B6 deficiency, the activities of α-fucosidase, β-glucuronidase, β-galactosidase acid phosphatase and hexosaminidase but not α-mannosidase increased in rat kidney (Diapaolo et al., 1975). In vitamin E deficient rat liver increased catheptic and arylsulphatase activities but not ribonuclease and β-galactosidase activities were noted (Zalkin et al., 1962).

Because of the great number of variables other than diminished tissue AA concentrations which must be considered with the acutely deficient guinea pig, a state of chronic ascorbic acid deficiency was induced. Previously, Ginter and co-workers (1967, 1976) demonstrated that guinea pigs chronically deficient in AA have tissue concentrations of AA similar to those observed in scorbutic animals while growth proceeded normally. This model was employed to examine the effects of diminished tissue AA concentrations on the activity of hexosaminidase, acid phosphatase, and cathepsin A and B in the absence of other effects seen in scurvy.

Guinea pigs fed 0.5 mg AA/100 g body weight/day had

low serum and brain AA concentrations (Figs. 4 and 5) similar to those observed in scorbutic animals (Table 1) but showed none of the clinical signs of scurvy (Plate 4) and grew at rates comparable to guinea pigs fed chow or 10 mg AA/day (Fig. 3). These animals were taken as biochemically deficient in AA as brain concentrations of norepinephrine were significantly lower than that of control animals fed chow 10 mg AA/day (Table 7; Appendix 5 and 6).

In guinea pigs receiving 0.5 mg AA/100 g body weight/day, an indication that AA may affect the activities of some lysosomal enzymes was noted. The specific activities of lysosomal hexosaminidase and acid phosphatase, but not cathepsins A and B, changed (Figs. 7, 8 and 9). During the period of AA supplementation (weeks 5 - 10) lysosomal acid phosphatase and hexosaminidase remained higher than that found in lysosomes isolated from control animals fed chow or 10 mg AA/day and was comparable to activities noted in deficient animals (Figs. 8 and 9, Table 2). This may be a specific response of these enzymes to low dietary availability of AA as no changes were observed with cathepsins A and B. Alternatively, the low amount of the vitamin

ingested may induce stress in these animals and thereby elevate the activities of these enzymes. Distinct populations of lysosomes have been identified in liver (Stahn et al., 1970) and lysosomes may have different enzyme complements which are affected to different degrees by factors such as marginal intake of a nutrient. Thus, the changes observed in lysosomal enzymes from guinea pigs chronically deficient in AA as well as in those acutely deficient in the vitamin (Table 5) may only reflect changes in specific populations of lysosomes.

Hexosaminidase and acid phosphatase were elevated at week 10 of the experiment ($P < 0.05$) (Figs. 8 and 9). This increase appeared to be maintained at week 12, but statistical analysis of the data indicated that the differences between the groups were not highly significant at week 12 ($P < 0.5$) (Appendix 3 and 4). This may reflect the variability observed in the group of 4 animals fed 0.5 mg AA/100 g body weight/day which masked the effects of AA intake. Alternatively, the decreased activity observed with hexosaminidase and acid phosphatase at week 12 could be due to adaption of these enzymes to low dietary availability of AA; adaption was observed for brain

norepinephrine and dopamine in guinea pigs receiving 1 mg AA/100 g body weight/day (Table 7). A study extending beyond 12 weeks with a greater number of animals would differentiate between these alternatives. Thus, in spite of diminished tissue AA concentrations and biochemical deficiency of AA, it cannot be equivocally stated (nor refuted) that AA regulated the activity of lysosomal hexosaminidase and acid phosphatase in vivo.

No basis for the increase in specific activity observed with some lysosomal enzymes (Table 5, Figs. 8 and 9) could be determined. Mixing experiments with tissue homogenates or subcellular fractions did not show the presence (or absence) of any inhibitor or activator molecules. Whether the changes in lysosomal hydrolase activity were the consequence of increased synthesis of enzymatic proteins, decreased degradation of enzymatic proteins, increased degradation of nonenzymatic proteins, or a combination of these was not determined.

Lysosomes isolated from livers of normal control guinea pigs, scorbutic guinea pigs and guinea pigs pair fed to scorbutic guinea pigs were used for in vitro studies examining the involvement of AA in lysosomal enzyme

regulation. These studies did not unequivocally support the hypothesis that AA was a regulatory molecule for lysosomal hydrolases.

Regardless of the AA status of the animal, the effects of in vitro AA supplementation were essentially the same. Physiological concentrations (2.5 mM) of AA inhibited lysosomal hexosaminidase somewhat (10%) whereas no more than 70% inhibition could be achieved with 500 mM (100 μmoles) AA (Fig. 10). The low amount of inhibition observed with physiological amounts of the vitamin and the high ratio of AA to lysosomal protein (8 μg of lysosomal protein was routinely used) detracted from any physiological role for AA in regulating lysosomal enzymes. Furthermore, the Ki of 0.18 M observed for hexosaminidase inhibition supported this conclusion (Figs. 12, 13 and 14).

AA is capable of reversible oxidation; and as a prerequisite for hexosaminidase inhibition was the presence of oxygen and metal ion (Table 13) these studies indicated that AA per se was not the inhibitory species. Studies employing specific scavengers of oxygen radicals suggested that monodehydroascorbic acid or some intermediate in the oxidation of AA to dehydroascorbic acid was responsible

for the inhibition. Thus, the high concentrations of AA required in vitro may be necessary to generate the actual inhibitory species and therefore, not represent the actual inhibition concentration.

Sugar lactones are known to specifically inhibit the corresponding glycosidase (Conchie and Levvy, 1957) and it was suggested that the inhibition of lysosomal glycosidases by sugar lactones and AA may be due to their lactone structure (Kanfer and Spielvogel, 1973). D-ascorbic acid and araboascorbic acid are lactones structurally similar to AA and these compounds also inhibited the enzyme (Table 10). Although D-ascorbic acid and araboascorbic acid are not naturally occurring in mammals, they may inhibit hexosaminidase because of their structural similarity to AA. However, glucoascorbic acid and dehydroascorbic acid are also lactones and these compounds failed to inhibit the enzyme (Table 10) indicating that the lactone structure alone was not responsible for the inhibition.

In view of the high concentrations of AA required to inhibit hexosaminidase in vitro (Fig. 10) and the Ki (0.18 M) of this inhibition, it was of interest to see if

lysosomes contained AA. Lysosomes were analyzed for AA content to see if AA was sequestered and enriched in these particles. AA was routinely analyzed by the 2, 4-dinitrophenylhydrazine procedure, the limit of sensitivity being 0.05 μmoles AA. Assuming physiological concentrations of the vitamin present in lysosomes, then 50 mg of lysosomal protein, the amount routinely obtained, would contain 0.125 μmoles AA. Analysis of liver homogenate and the 100.000 x g supernatant of this homogenate showed that all the AA was present in the supernatant and none associated with the lysosomes. This may be expected because the lysosomal membrane is permeable to small molecules of molecular weight $\leq$ 300 (Desnick et al., 1976). Lysosomes were isolated in the presence of physiological concentrations of AA (2.5 mM) to prevent the extraction of any AA which may have been present in lysosomes during the isolation procedure. However, no AA was found to be associated with these particles. AA from the medium may be expected to penetrate the lysosomal membrane, but this seemed not to occur. It may be that AA was rapidly degraded in the lysosomes. Alternatively, the lysosomal membrane may not be permeable to AA or the concentration of AA in the medium

not high enough to force AA into the lysosomes. Furthermore, uptake of AA by lysosomes may be an active process. Uptake of AA into retina or brain cortex or ovary slices in vitro appeared to require an active transport mechanism which was not required for liver (Hornig, 1975). However, studies measuring the intralysosomal pH of macrophages indicated that low intralysosomal pH was maintained in part by an active energy requiring mechanism (Ohkuma and Poole, 1978). Thus, disruption of cells and isolation of cellular organelles may destroy any AA sequestering mechanism.

Because of the high Ki for AA inhibition of hexosaminidase, it is difficult to understand how concentrations of AA 100 times that present in the cell could be accumulated in lysosomes. Recent studies with coated vesicles isolated from brain indicate that these vesicles can accumulate calcium by an ATP-dependent process 10 - 20 fold higher than the amount present in the medium. Furthermore, in the presence of potassium oxalate, a calcium trapping agent, the calcium concentration was increased by an additional 5 - 10 fold. Whether a similar situation may be found in the lysosomes is not

known at this time.

Although the biochemical processes associated with the lysosomes are well known, the underlying regulating processes are still largely undefined. The results from in vivo and in vitro studies undertaken to assess the involvement of AA in lysosomal enzyme regulation do not allow any unequivocal conclusions to be drawn at this time.

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Appendix Table 1

Analysis of Variance: In vivo effects of chronic ascorbic acid deficiency on guinea pig lysosomal hydrolase activities: cathepsin A activity weeks 5-10 and week 12

	Source of Variance	df	SS	MS	F-value	P[1]
Weeks 5-10	Diets	3	1653	551	1.47	NS[2]
	Weeks	5	4327	866	2.32	NS[2]
	Error	15	5605	374		
	Total	23	11586			
Week 12	Animals	3	1654	551	0.13	NS
	Diets	2	716	358	0.09	NS
	Error	6	24511	4085		
	Total	11	26882			

1 Probability of chance occurrence.

2 Not significant.

Appendix Table 2

Analysis of Variance: In vivo effects of chronic ascorbic acid deficiency on guinea pig lysosomal hydrolase activities: cathepsin B activity weeks 5-10 and week 12

	Source of Variance	df	SS	MS	F-value	P[1]
Weeks 5-10	Diets	3	1880	627	1.27	NS[2]
	Weeks	5	3251	650	1.31	NS
	Error	15	7422	495		
	Total	23	12553			
Week 12	Animals	3	2891	964	1.55	NS
	Diets	2	2926	1463	2.35	NS
	Error	6	3732	622		
	Total	11	9548			

1 Probability of chance occurrence.

2 Not significant.

Appendix Table 3

Analysis of Variance: In vivo effects of chronic ascorbic acid deficiency on guinea pig lysosomal hydrolase activities: hexosaminidase activity weeks 5-10 and week 12

	Source of Variance	df	SS	MS	F-value	P[1]
Weeks 5-10	Diets	3	2917378	972459	16.48	< 0.01
	Weeks	5	1080093	216018	3.66	< 0.05
	Diet x weeks	15	2385693	159046	2.70	< 0.05
	Residual	24	1416008	59000		
	Total	47	7799173			
Week 12	Animals	3	1709820	569940	0.92	NS[2]
	Diets	2	3181560	1590980	2.58	NS
	Error	6	3699445	616574		
	Total	11	8590825			

[1] Probability of chance occurrence.

[2] Not significant.

Appendix Table 4

Analysis of Variance: In vivo effects of chronic ascorbic acid deficiency on guinea pig lysosomal hydrolase activities: acid phosphatase activity weeks 5-10 and week 12

	Source of Variance	df	SS	MS	F-value	P[1]
Weeks 5-10	Diets	3	744227	248076	3.74	< 0.05
	Weeks	5	694131	138826	2.10	NS[2]
	Diet x weeks	15	912672	60845	0.92	NS
	Residual	24	1589821	66243		
	Total	47				
Week 12	Animals	3	442800	147600	1.16	NS
	Diets	2	757992	378996	2.99	NS
	Error	6	1961878			
	Total	11				

1 Probability of chance occurrence.

2 Not significant.

Appendix Table 5

Analysis of Variance: In vivo effects of chronic ascorbic acid deficiency on dopamine concentrations in guinea pig brain: weeks 5-10 and week 12

	Source of Variance	df	SS	MS	F-value	P[1]
Weeks 5-10	Diets	3	49170	16390	14.34	< 0.001
	Weeks	5	1962	393	0.34	NS[2]
	Error	15	17140	1143		
	Total	23	68272			
Week 12	Animals	3	2216	739	0.98	NS
	Diets	2	29465	14732	19.50	< 0.01
	Error	6	4534	756		
	Total	11	36215			

1 Probability of chance occurrence.

2 Not significant.

Appendix Table 6

Analysis of Variance: In vivo effects of chronic ascorbic acid deficiency on norepinephrine concentrations in guinea pig brain: weeks 5-10 and week 12

	Source of Variance	df	SS	MS	F-value	P[1]
Weeks 5-10	Diets	3	52066	17355	23.92	< 0.01
	Weeks	5	4545	909	1.25	NS[2]
	Error	15	10882	725		
	Total	23	67493			
Week 12	Animals	3	3536	1179	0.96	NS
	Diets	2	22604	11302	9.19	< 0.05
	Error	6	7379	1230		
	Total	11	33519			

[1] Probability of chance occurrence.

[2] Not significant.

Electron microcopy report of hepatic and brain tissue of normal control, scorbutic and starved control guinea pigs.

Health Sciences Centre
CHILDREN'S CENTRE

Lab. No. [illegible]

PATHOLOGY REPORT

Name KANFER, Scorbutic Guinea Pigs Age

Doctor Dr. Kanfer Ward

Provisional Diagnosis Hosp. No.

Specimen Liver specimens Date Received December 6, 1976

CLINICAL NOTES:

Robert J. Gourley Laboratory
Electron Microscopy Report

Liver specimens from six guinea pigs were received for ultrasound examination. These were received labelled as follows: 1a, 1B, C2, 3a, 3b, 3B.

The hepatocytes in a normal liver can exhibit a prominent variation in ultrastructural appearance. For example, adjacent hepatocytes within the same liver may exhibit a noticeable variation in glycogen content, lipid content, smooth and rough surfaced endoplasmic reticulum and secondary lysosomes.

The livers of these six animals are microscopically and ultrastructurally normal. Although there is some ultrastructural variation in the small samples studied, it is no greater than would be anticipated in six different normal animals or even in different areas from the same liver.

Date Received January 4, 1977

Liver specimens of nine guinea pigs were received for ultrastructural study. These were labelled: 1B, 2B, 1D, 2D, 3B, 1C, 2C, 3D, and 3C. There is, in addition, a sample of brain tissue from animal 3C.

The hepatocytes of these nine animals exhibit some variation in glycogen content, lipid content, and secondary lysosomes, but this is no more than I would regard as normal variation. There is a similar variation in the relative content of smooth and rough surfaced endoplasmic reticulum; this variation is again within normal limits.

Microbodies or peroxisomes are present in the hepatocytes of all nine animals, and are morphologically and numerically similar in all animals. These are smaller than mitochondria, possess a matrix of approximately similar density to that of mitochondria, and are encompassed by a membrane similar to that of smooth endoplasmic reticulum. In a few, this enveloping membrane appears to be continuous with that of cisternae of endoplasmic reticulum. A few structures of similar size, have no apparent enveloping membrane, and resemble small condensations of cytoplasmic matrix. Some microbodies contain a small dense body or nucleoid. Microbodies and primary lysosomes cannot be differentiated on morphological grounds alone.

"Secondary" lysosomes (probably autophagosomes) are present in the hepatocytes of one animal (2D). These consist of accumulations of concentric membrines, material of variable electron-density, and sometimes a mitochondrion, segregated within a space, usually bounded by a membrane.

Lipid droplets, when present, lie free within the cytoplasm with no enveloping membrane. These are apparent in the hepatocytes of three animals (2C, 2D, and 3D).

Glycogen particles were not identified in any of these preparations. Cacodylate buffer was utilized in the processing of these tissues. We have recently discovered that the utilization of this buffer may prevent the ultrastructural demonstration of glycogen particles. On the assumption that abundant clear cytoplasmic matrix is most probably a reflection of high glycogen content, I have assumed that there is abundant cytoplasmic glycogen in the hepatocytes of five animals (1B, 3B, 1C, 3C, and 1D).

Apart from some degree of artefactual distortion, grey and white matter of the brain of animal no. 3C is ultrastructurally normal.

cont'd....

Health Sciences Centre
CHILDREN'S CENTRE

Lab. No. [illegible]-6

PATHOLOGY REPORT

Name KANFER, Scorbutic guinea pigs Age

Doctor Dr. Kanfer Ward

Provisional Diagnosis Hosp. No.

Specimen Liver, brain Date Received January 10, 1977.

CLINICAL NOTES:

ROBERT J. GOURLEY LABORATORY
ELECTRON MICROSCOPY REPORT

Specimens of liver and brain from two guinea pigs were received for ultrastructural examination. These specimens were labelled as follows:

1a - Liver
2a - Liver
1a - Brain
2a - Brain

The liver of these two animals is microscopically and ultrastructurally normal, with no significant ultrastructural difference in the two animals.

Apart from some degree of artefactual ultrastructural distortion, the samples of grey and white matter of brain are microscopically and ultrastructurally normal.

There is no apparent significant difference in the ultrastructural appearance of these viscera in the two animals.

Jan Hoogstraten.

Jan Hoogstraten, M.D., Ph.D.,
Pathologist

JH/sk
cc. Dr. Julian Kanfer, Dept. of Biochemistry,
Basic Science Building

85-248

Key: Samples prefixed by 1 or 2 or 3 represent normal control or scorbutic or starved control guinea pigs, respectively.