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# INVOLVEMENT OF LYSOSOMES IN SPONTANEOUS ATHEROGENESIS AND THE EFFECTS OF ESTROGENS

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# INVOLVEMENT OF LYSOSOMES IN SPONTANEOUS ATHEROGENESIS AND THE EFFECTS OF ESTROGENS

BY

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DISSERTATION

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ABSTRACT

INVOLVEMENT OF LYSOSOMES IN SPONTANEOUS ATHEROGENESIS AND THE EFFECTS OF ESTROGENS

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Alterations and accumulations of glycosaminoglycans (GAG) and lipids are prominent features in atherosclerotic lesions. Lysosomes are essential for cellular catabolism and are known to be altered in advanced atherosicerotic lesions; however, their involvement in spontaneous atherogenesis or lesion progression is unclear. Estrogens have been reported to interact with lysosomes and are thought to provide a "protective" effect against development of atherosclerotic lesions. Analysis of lysosomes from sites predisposed to lesions, as well as consideration of the effects of estrogens on lysosomes at different stages of lesion development, may provide insight into biochemical mechanisms of spontaneous atherogenesis and lesion progression. Simultaneous analysis of lesion-resistant aortic segments provides a control for differentiating between aging processes and lesion development.

Lysosomal fragility and marker enzyme activities, N-acetyl-B-hexosaminidase (NAHase) and acid phosphatase (APase), were measured in subcellular fractions from upper thoracic aortas (lesion-resistant areas) and celiac bifurcations (site predisposed to lesions) from

atherosclerosis-susceptible White Carneau (WC) and atherosclerosis-resistant Show Racer (SR) pigeons at 1 day, 6 weeks, 6 months, and 6 years of age. Isolated arterial segments were also incubated with 17B-estradiol at physiological temperatures to determine the effect on lysosomal fragility and enzyme activities.

Lysosomal enzyme activities and protein yields in both aortic sites from 6 week old WC pigeons were higher than in corresponding SR; thus, turnover rates of cellular components would appear higher in the WC than in the SR. However, the lysosomes from 6 week old WC aortas also appeared more fragile than lysosomes from SR aortas. Since this is prior to appreciable GAG and lipid accumulation in WC celiac sites, excessive release of lysosomal enzymes and/or greater activities of these enzymes may cause alterations in connective tissue matrix and mitochondrial function which are associated with lipid accumulation during atherogenesis in the WC. The increased lysosomal fragility may also deplete the cell's vacuole system of acid hydrolases therefore decreasing catabolism of intracellular components and endocytosed material eg. lipid.

Estrogen treatment increased APase activity and protein yield in the "lysosomal" fraction of 6 week old birds suggesting an increase in number of lysosomes which may aid cellular catabolism and explain the reported estrogen "protection" against lesion development in WC.

Soluble NAHase activity increased in the WC celiac segment by 6 years of age and may explain the altered GAG profiles which occur during atherosclerotic lesion progression.

Since the greater lysosomal enzyme activities, protein yields and membrane fragility in the WC occur at both tissue sites, a genetic mechanism is indicated. In the WC celiac segment higher lysosomal enzyme activities, protein yields and membrane fragility appear augmented by local factors, and may play a major role in spontaneous atherogenesis.

# I. INTRODUCTION

Atherosclerosis is implicated in nearly 90% of all cardiovascular disease, which is the primary cause of death in much of the industrialized world (Klevay, 1975; Ross, 1975). In 1969, the World Health Organization proclaimed cardiovascular disease the most decimating disease ever to affect mankind (Stamler, 1970). Atherosclerosis has been found in arteries of 16th century B.C. mummies, yet the initiating factors of the disease are still poorly understood.

Many animal species which develop spontaneous or experimentally induced atherosclerosis have been used as models to study initiating, progressive, and regressive factors in the disease. One of the few animal models which develops spontaneous atherosclerosis under normal conditions without cholesterol feeding or induced arterial injury is the White Carneau pigeon.

Spontaneous atherosclerosis in pigeons and its close resemblance to the human disease was first described by Clarkson, et al. (1959). Santerre, et al. (1972) later described the normal and atherosclerotic architecture and topography of the muscular foci at the celiac bifurcation in prenatal to six-year old White Carneau and Show Racer pigeon aortas. In this model system, which includes a negative-control, atherosclerotic susceptibility, lesion site specificity, lesion severity, and rate of disease progression are quite predictable, Since the White Carneau (WC) pigeon shows a greater tendency than the Show Racer (SR) pigeon to develop atherosclerosis

under identical conditions of environment, exercise, and diet, the disease in pigeons is believed to have a genetic basis (Prichard, et al., 1962; Thomas et al., 1973).

By four years of age nearly 100% of all WC have grossly visible spontaneous lesions in the innate muscular cushion at the celiac bifurcation (Clarkson et al., 1965; Cooke and Smith, 1968; Lindsay and Nichols, 1971; Prichard et al., 1964; Santerre et al., 1972). It has been suggested that these raised muscular cushions are the counterpart of the musculo-elastic layers of intimal thickening in the human lesions which is a prerequisite for lipid accumulation (Cooke and Smith, 1968; Santerre et al., 1972). The negative-control (SR) in the pigeon model has facilitated numerous in vivo and in vitro comparisons of cellular and intercellular constituents, as well as metabolic capabilities of atherosclerotic and non-diseased pigeon aortas (Curwen and Smith, 1977; Lofland and Clarkson, 1959, 1965; Lofland et al., 1967; Nicolosi et al , 1972; Smith et al., 1965; Smith et al., 1966; Hajjar and Smith, 1978, 1980; Zemplenyi and Rosenstein, 1975; Hajjar et al., 1980). Furthermore, the WC-SR system permits discrimination between atherosclerotic events and normal developmental or aging processes.

Studies of atherosclerosis in man and a variety of animal models has led to the hypothesis that the mechanism(s) inciting the disease is a normal arterial repair process or a non-specific mesenchymal response to irritating and injurious factors (hypertension, emotional stress, diet, hormones, or autoimmune irritants). However, it is unclear how the associated proliferative and degenerative events affect arterial homeostasis. These events in-

clude intimal accumulation of cells, structural proteins, complex carbohydrates and lipids. In contrast to results with many experimental animal models elevated blood lipid and cholesterol levels or aberrant liproprotein profiles do not seem to play a role in the development of spontaneous atherosclerosis in the WC pigeon (Jensen et al., 1978; Lofland and Clarkson, 1959; Wagner et al., 1973).

It has been well documented that the lysosomes of arterial smooth muscle cells are altered in atherosclerosis and other vascular diseases. Increased numbers of lysosomes, including lipid-containing lysosomes, have been demonstrated by both cell fractionation and ultrastructural cytochemical techniques in aortic cells: from cholesterol-fed rabbits (Peters and de Duve, 1974; Shio et al., 1974; Haley et al., 1977); from humans with both atheromatous lesions (Berberian and Fowler, 1979) and areas of fibromuscular thickening without focal lipid accumulation or superficial fatty streaks (Coltoff-Schiller et al., 1976); and, from hyperlipemic monkeys (Goldfischer et al., 1975). Biochemical analyses of atheromatous and hypertensive aortas have also demonstrated that the activity of lysosomal acid hydrolyses were significantly elevated when compared with normal vascular tissue (Berberian and Fowler, 1978; Wolinsky et al., 1974; Wolinsky et al., 1975; Peters et al., 1973; Haley et al., 1977). Peters et al. (1973), have further shown in cholesterol-fed rabbits that lysosomal membrane stability increases with the severity of atherosclerosis.

Lysosomes hydrolyze both extracellular material incorporated

into the cell by endocytosis, and cytoplasmic elements sequestered within the lysosome by autophagia (de Duve, 1969). The release of lysosomal enzymes extracellularly is also considered to play a major role in the physiological as well as pathological breakdown of extracellular macromolecules (Reynolds, 1969). Alterations in lysosomal enzymes or in the lysosomal membrane may alter normal catabolic activity in the arterial wall either by a decrease in normal degradative processes or by abnormal release of enzymes causing alterations in the structural architecture of the vessel. A metabolic defect in arterial wall catabolism may be a causative factor in atherogenesis or may be a consequence of an earlier initiating factor.

A sex difference in suscepitibility to atherosclerosis has been well documented in humans. Women prior to menopause show a lower incidence of heart attacks than men in the same age group. Ovariectomy or menopause cause the loss of this "resistance" to coronary events, and estrogens are thought to provide the protective effect. Because of the protective effect of estrogens studies were initiated in which estrogens were administered to men who had had previous myocardial infarctions or prostatic carcinoma (Stamler et al., 1956; Coronary Drug Project, 1970; Vet. Admin. Cooperative Urp. Res. Grp., 1967). The results from these studies are conflicting, and many variables may have affected the outcome. Although women have slightly lower serum cholesterol; levels of estrogens needed to produce alterations in serum cholesterol in men are relatively high and beyond the physiological range. Such high doses, especially of synthetic estrogens (i.e., ethinyl

estradial is 60 to 75 times as potent as conjugated estrogens), were found to increase the number of myocardial infarctions (Coronary Drug Project, 1970; Vet. Admin. Cooperative Res. Group, 1967; McGill and Stern, 1979). However, administration of low doses of conconjugated estrogens to men tended to increase the survival rate over a five year study (Stamler et al., 1963). On the other hand, McGill and Stern (1979) concluded that estrogens do not cause regression of atherosclerosis in men; men who have taken estrogens for several years have a higher percentage of fibromuscular lesions than lipid-laden lesions (McGill and Stern, 1979). Perhaps estrogens are able to stimulate mechanisms for the removal of lipid material. Similarly, administration of conjugated estrogens to five to seven year old WC pigeons did not affect the actual size of the spontaneous aortic lesions; however, the amount of lipid material found within the lesions was significantly lower when compared to the controls (Hanash et al., 1972). Hanash et al. (1972) also reported a significant difference between sexes within the control and estrogen treated groups (female aortic lesions always contained less lipid material). Souadjian et al. (1968) found that administration of conjugated estrogens to immature WC pigeons significantly decreased the development of atherosclerotic lesions. Therefore, it appears that in both man and pigeons administration of estrogen to subjects with pre-existing atherosclerotic lesions does not cause regression but does appear to aid in the removal of lipid from the lesions. The presence of estrogens prior to lesion development does appear to prevent or retard the development of lesions.

The effects of estrogens on atherosclerosis in other experi-

mental animals has been shown to vary with species and location of lesions in the vascular tree, as well as with the type of disease (spontaneous vs. induced) (Hanash, et al., 1972; Sirek, et al., 1977; Prichard, et al., 1966; Stamler, et al., 1956). Differences in hormone receptors in the arterial wall may account for differences in the mesenchymal response. Autoradiographic studies using $^{3}H$ -estradiol have demonstrated receptors for estrogens not only in "target organs", but in blood vessels (Stumpf and Sar, 1977) and cardiac muscle as well (Stumpf et al., 1977).

Administration of estrogens to animals has been shown to alter lysosomal acid hydrolase activity: decreasing the abnormally high aortic enzyme activity in hypertensive rats (Wolinsky, et al., 1974); increasing activity in rat myometrium (Sloane and Bird, 1977); and, decreasing activity in the pituitary and hypothalamus of male frogs (Milone, et al., 1978). Furthermore estrogens have been shown to interact with lysosomes in vivo and in vitro; however, the effects are equivocal. Several studies have reported that estrogens have a labilizing effect on lysosomes (Szego, et al., 1971; Szego, et al., 1977; Briggs, 1973; Sergeer, et al., 1978) while others have reported a stabilizing effect (Bodel, et al., 1972; Weissman, 1969). Increased stabilization of membranes has been correlated with decreased ability of the membrane to fuse with other membranes (Papahadjopoulos, et al., 1973). Likewise a decrease in membrane stability (or increase in fragility) would increase the ability of the membranes to fuse. If lysosomes are stabilized in atherosclerotic lesions, then their ability to fuse with endocytotic vesicles,

autophagic vesicles, the plasma membrane or secondary lysosomes (to provide additional degradative enzymes) would be diminished thereby decreasing the catabolic capacity of the cell and cause subsequent accumulation of connective tissue and lipids. A decrease in the lysosomal membrane stability (possibly by estrogens) would prevent this cascade of events. On the other hand, lysosomes whose membranes are destabilized can also have a deleterious effect on the cell through excessive extra- and intra-cellular release of lysosomal hydrolases. Since the effect of cholesterol on membrane fluidity depends on the amount of cholesterol present, as well as the overall composition of the membrane, perhaps the conflicting reports of the effects of estradiol on membrane stability may be caused by differences in composition of various membranes studied.

Although no significant sex differences in the suscepitibility of pigeons to atherosclerosis have been seen (Santerre, et al., 1972; Hajjar, et al., 1980), estrogens have still been shown to produce a protective or preventive effective in immature WC pigeons (Souadjian, et al., 1968). However Koes, et al. (unpublished) have reported greater vacuolization and amounts of lipid in cell cultures from the aortas of male pigeons than in cell cultures from the aortas of female pigeons. The lack of a sex difference in the development of atherosclerotic lesions in the WC may be due to their reproductive behavior (Brannigan, 1973). The WC males are more aggressive than the SR males; therefore, the WC reproduce more readily. The WC also wean their young earlier and start a second hatch sooner than the SR pigeons. These findings suggest a difference in either the balance of circulating hormones or in the response of the male birds to hormones. Brannigan (1973) also found

adrenal hyperplasia in WC pigeons (both sexes) which increased when the birds were isolated or grouped (instead of paired). Lutmer and Wexler (1970) have shown that repeatedly bred female mice develop spontaneous atherosclerosis and have high adrenocortical activity. Furthermore Iams and Wexler (1977) found that early weaning of the young increased the severity of the disease in the mothers, and that adrenalectomy inhibited the development of arteriosclerosis. Also, Friedman et al., (1972) showed that humans of a high coronary risk group had significantly elevated levels of corticosteroids. Szego (1972) has also shown that corticosteroids antagonize estrogen action in target tissues. Furthermore, androgen and glucocorticoid secretion by the adrenals occurs simultaneously (James, 1975), so that increased glucocorticoid secretion means increased androgen secretion as well. McMullin et al., (unpublished) found that the addition of an androsterone increased lipid vacuolization in smooth muscle cells cultured from WC aortas. Androsterone is metabolite of androstenedione which is one of the major androgens secreted by the adrenals (James, 1975). Therefore, the WC pigeon is hormonally as well as genetically disposed toward the development of atherosclerosis. This behavior pattern in the WC female may eliminate or oppose any "protective" effect of estrogens, and enhance atherogenesis through androgen and/or glucocorticoid effects on aortic smooth muscle cell metabolism.

## II. THE PRESENT STUDY

Although the protective effect of estrogens in cardiovascular disease has been hypothesized for several decades, the mode or modes of action remain to be elucidated. A major complication in the elucidation of estrogen mechanisms is the apparent diversity of tissue responses, depending on the dose of estrogen used, the location and type (spontaneous vs. cholesterol-fed) of lesion, and the age and hormonal status of the recipient. Estrogens are known to interact with lysosomes, and alterations in lysosomes are common features of various blood vessel lesions. Therefore, the present study was undertaken to determine whether there is a correlation between lysosomal alterations, estrogen action and atherogenesis. Since the WC pigeon develops spontaneous atherosclerotic lesions which are site and age specific, morphologically similar to human lesions, and appear to respond to estrogen in a similar manner, it is an ideal animal model for the study of atherosclerosis and its initiating factors, as well as for examining the effect of estrogens. The simultaneous comparison of the atherosclerosis-resistent SR as a control enables the investigator to discriminate between differences due to age, vascular location and atherosclerosis.

This study was designed with the following as independent variables:

Two breeds - WC and SR;

Two Sexes - male and female;

Four Ages - 1 day, 6 weeks, 6 months, and 6 years;

Two Aorta Sites - Upper thoracic and celiac bifurcation;

Two treatments - control and estrogen;

Four Tissue Fractions - crude homogenate, lysosomal, microsomal, and soluble fractions.

Two Activities - Brij-35 and Free.

The above scheme contained two separate dependent variables:

N-Acetyl-B-hexosaminidase (NAHase) and Acid Phosphatase (APase) activities.

Each cell in the two designs contained results from 3 replicate tissue pools, except for 1 day aortas where aorta sites were not separated.

Activities of the above lysosomal marker enzymes, latency of these enzymes were determined in the upper thoracic aorta (non-lesion site) and in the muscular cushion at the celiac bifurcation (pre-disposed lesion site in WC) in 1 day old (pre-atherosclerotic), 4-6 week old (pre-atherosclerotic, just weaned), 4-6 month old (early biochemical atherosclerosis), and 5-6 year old (advanced atherosclerosis) WC and SR pigeons. In vitro incubation of tissues with 17B-estradiol enabled measurement of any direct effect of estrogen on lysosomes and lysosomal enzymes.

N-Acetyl-B-hexosaminidase is considered to be the most reliable lysosomal marker enzyme and has not been demonstrated in other subcellular organelles. Acid phosphatase is the most commonly used lysosomal marker enzyme even though activity has been demonstrated

in the endoplasmic reticulum. Enzyme activities were measured in the crude tissue homogenate, the lysosomal-mitochondrial pellet ("lysosomal" fraction), the microsomal pellet, and the soluble fraction (105,000 g supernatant). Further separation of lysosomes from mitochondria was not necessary since the enzyme activities measured have not been demonstrated in mitochondria. The enzyme activity present in the microsomal fraction is of interest since ultrastructural alterations of the endoplasmic reticulum (ER) and increased B-glucuronidase activity in the ER have been demonstrated in smooth muscle cells cultured from WC aortas (Wight, et al., 1977; Smith and Smith, in preparation). The soluble enzyme activities were also measured in order to determine non-menbrane bound enzyme.

The percentage of latent enzyme activity (enzymes contained in intact lysosomes and not available to the substrate until the lysosomes are disrupted) was determined in the following manner. The enzyme activity was measured before and after the lysosomes were disrupted by the addition of a non-ionic detergent, Brij-35, to the assay mixture. The free enzyme activity (activity prior to lysosomal rupture) is then subtracted from the total enzyme activity (activity after lysosomal rupture) and the value is divided by the total activity:

$$\% \text{ Latent enzyme activity} = \frac{\text{Total activity} - \text{Free activity}}{\text{Total activity}} \times 100$$

Tissue samples of birds of opposite sexes were kept segregated because previous studies in other animals have shown that APase and NAHase enzyme activities were sex dependent. The response of aortic tissue to estrogen may vary with sex as well.

## III. MATERIALS AND METHODS

### Tissue Samples

One-day old White Carneau and Show Racer pigeons were obtained from colonies maintained at the University of New Hampshire. These colonies were established with pigeons from stock obtained from the Palmetto Pigeon Plant (Sumter, S.C.). These birds are of the same lines as described by Clarkson et al.,(1959). Six-week, six-month, and six-year old WC and SR pigeons were obtained from either the Palmetto Pigeon Plant or from the U.N.H. colonies. The birds were fed Palmetto Health Grit and Purina Pigeon Choe Crackers, or a mixture of whole grains: wheat peas, Kaffir, and milo, ad libitum.

Pigeons were sacrificed by exsanguation, and aortas from the arch to just below the celiac bifurcation were removed and placed in ice-cold Hank's Balanced Salt Solution containing glucose (H+). Thoracic and celiac segments were separated as described by Santerre, et al. (1972). The tissues were dissected free of blood and extraneous material, and then the adventitia was removed according to the mehtod of Wolinsky and Daley (1971). Thoracic and celiac segments (approximately equal in size) were subsequently pooled respectively to provide sufficient tissue for analysis (20 mg samples from 2 birds per pool).

Each tissue pool was divided into two aliquots (tissue fragments of approximentely equal size), A and B, and weighed. Tissue aliquot A was incubated for 30 min. at physiological temperature (42C)

in H+ (100 ul/mg tissue) containing 0.5 ul/ml of 0.1 M phosphate buffer (pH 7.0) and 0.1% Bovine gamma globulin fr. II (buffer I). Aliquot B was treated similarly except that Buffer I contained 200 mg/ml of 17B-estradiol (final concentration of 100 pg/mlH+) in addition. After incubation the tissue was washed once with cold H+ and twice with cold 0.25M sucrose, and put on ice. There was no appreciable difference in NAHase activity between untreated tissue incubated at 42°C for 30 min. and tissue kept on ice for 30 min.

Subsequent to incubation and washing, the tissue was minced and homogenized in 0.25 M sucrose by 25 strokes of a Dounce homogenizer (Wheaton, pestle size A) at a concentration of 16 mg wet weight per ml sucrose. All glassware used was previously siliconized using Aqua-sil (Pierce, Inc.).

The tissue homogenate was centrifuged for 5 min. at 100g to remove debris and the supernatant divided into three aliquots. One aliquot (200 ul) was frozen for DNA analysis. The second aliquot (100 ul) was put on ice for subsequent enzyme analysis, while the third aliquot (500 ul) was separated into "lysosomal", microsomal, and soluble fractions by further ultracentrifugation (Beckman model L ultracentrifuge).

Samples were centrifuged at 800g for 10 min. to remove the nuclear fraction. The post-nuclear supernatant was then centrifuged at 20,000 g for 30 min. The pellet ("lysosomal" fraction) was resuspended in 0.25 M sucrose to the original volume (300 ul) and divided into two aliquots (150 ul each). One aliquot was frozen for protein analysis and the other kept on ice for subsequent en-

zyme analysis. The post-lysosomal supernatant was further centrifuged at 105,000 g for one hour. The 105,000 g pellet (microsomal fraction) was resuspended in 0.25 M sucrose to its original volume (200 ul) and kept on ice. The post-microsomal supernatant (soluble fraction) was also kept on ice for subsequent enzyme analysis.

A sample of 30 ul from each fraction was diluted 1:2 with either 0.25 M sucrose (free activity) or with 0.25 M sucrose containing 0.1% Brij-35 (Fisher) (total activity).

## Lysosomal Enzyme Analyses

Acid phosphatase and N-acetyl-B-hexosaminidase were determined in a similar manner using 4-Methylumbelliferyl derivatives as substrates at a final concentration of 4 mM (Barrett and Heath, 1977). Acetate buffer, 0.2 M (pH 4.8) and 0.1 mM citrate buffer (pH 4.1) were used for APase and NAHase analyses respectively. The substrate concentration and pH were optimized for each enzyme (Fig. 1a and 1b) and was the same for both breeds of pigeons and for both tissue sites.

Twenty ul of each sample to be assayed was incubated with 20 ul of the appropriate buffer solution for twenty minutes. The reaction was stopped by the addition of 1.7 ml of 0.5 M Carbonate buffer (pH 10.5),and the quantity of product formed was determined fluorometrically (Turner Model 430 Spectrofluorometer; excitation 365 nm; emission 450 nm) from a standard curve for 4-methylumbelliferone. The reactions were linear with time (20 min.) for both breeds of

Figure 1a

N-Acetyl-B-Hexosaminidase (NAHase) and Acid Phosphatase (APase) pH Curves

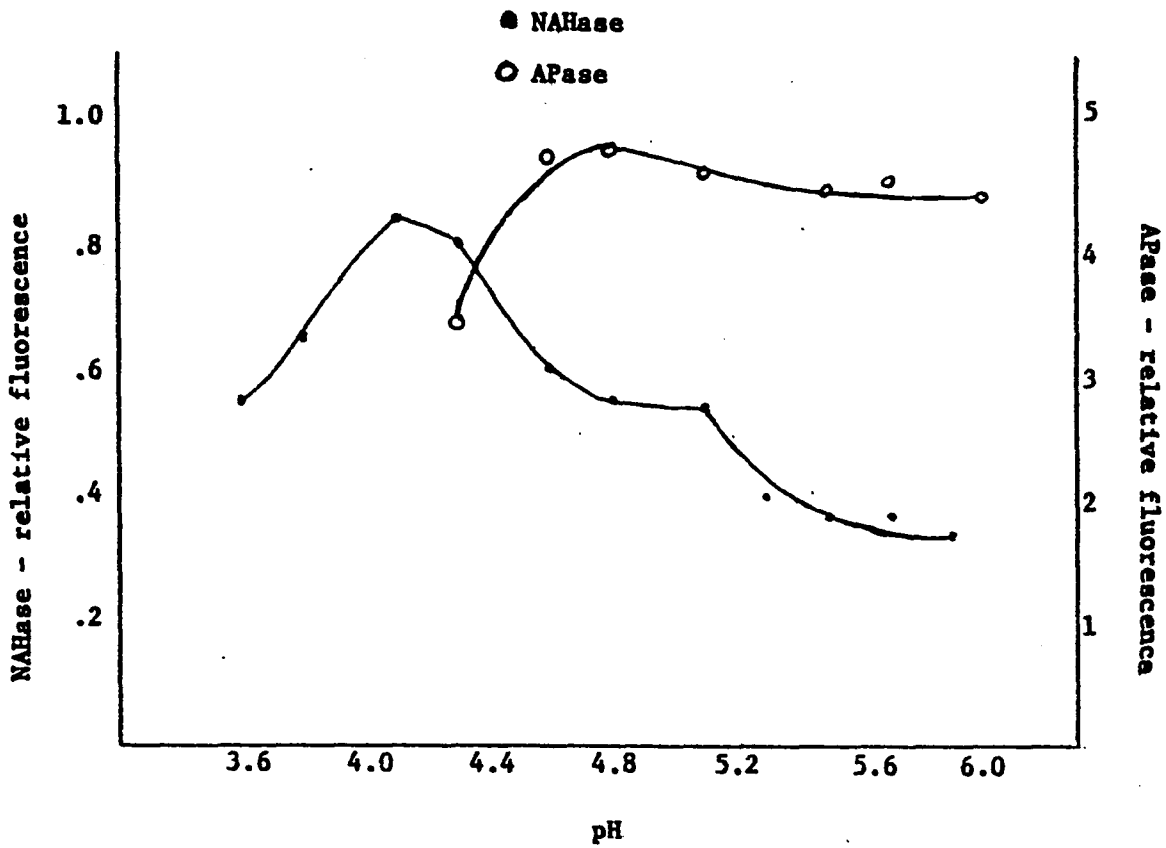
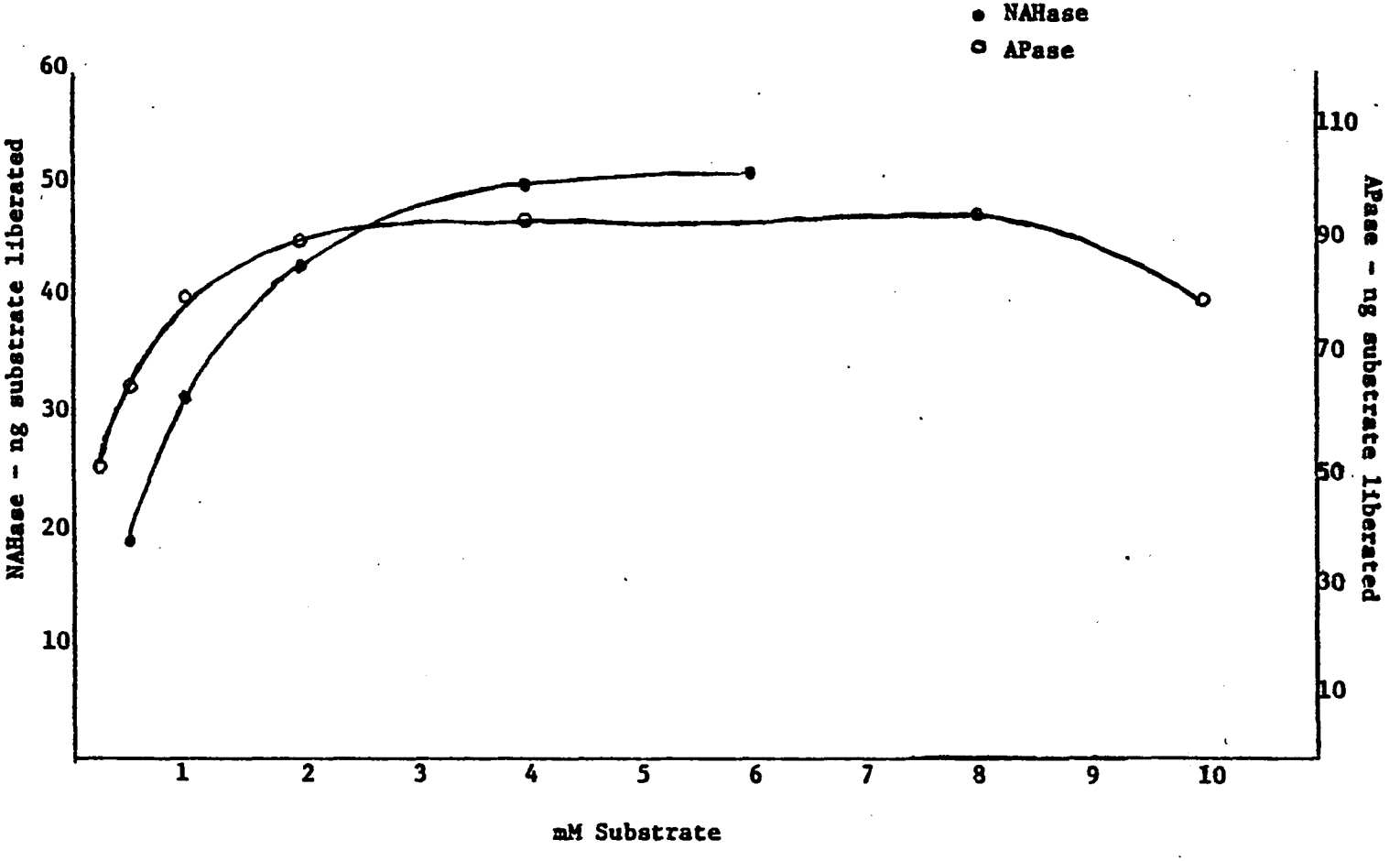


Figure 1b

N-Acetyl-B-Hexosaminidase (NAHase) and Acid Phosphatase (APase) Substrate Curves



pigeons and for both tissue sites at all ages.

## DNA Analysis

Duplicate 60 ul aliquots of the post 100g supernatant were analyzed for DNA content by the microfluorometric method of Kissane and Robins (1958), using 3,5-diamino-benzoic acid dihydrochloride, as modified for the Turner Model 430 Spectrofluorometer. Samples were frozen for not more than one month prior to analysis. Preliminary experiments determined that pronase digestion or sonication was not necessary for the complete release of DNA from the tissue; therefore, neither pronase digestion nor sonication was used prior to DNA analysis.

## Protein Analysis

Protein was quantitated by the protein-dye binding method of Bradford (1976). Aliquots of 40 and 70 ul of the "lysosomal" and microsomal fractions, respectively, were diluted to 0.4 ml with 0.25 M sucrose, and 0.1 ml of the dye reagent (0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid) provided in a test kit by Bio-Rad Laboratories was added. After twenty minutes samples were read at 595 nm on a Beckman model 26 spectrophotometer, and the quantity of protein determined from a standard curve for bovine serum albumin (Miles Laboratories).

## Development of Methods

### Determination of optimal conditions for lysosomal disruption

Membrane disruption with Brij-35 was found to liberate the greatest enzyme activity from the "lysosomal" fraction when compared to other methods of membrane disruption. Other membrane disruption methods evaluated included digitonin, freezing-thawing, hypotonic (0.05 M sucrose) solution, Triton x-100, sonication, and heating and cooling. Brij-35 was tested using concentrations from 0.001% to 8%, and a concentration of 0.1% produced the greatest effect. Enzyme activity increased with increasing Brij-35 concentrations up to 0.1% at which point the enzyme activity declined when the Brij-35 concentration was increased further.

### Determination of estrogen incubation time

Uptake of 17B-[6,7-$^{3}$H(N)]-estradiol (NEN) by the upper thoracic aorta and celiac bifurcation *in vitro* plateaued between 10 and 20 min. and remained relatively constant for up to 90 min. However, since NAHase activity was found to decrease when tissue was incubated for more than 45 minutes, 30 minutes was used as the optimal incubation time. Radioactivity in the tissue samples was determined by liquid scintillation spectrometry (Beckman LS 7000) after overnight digestion at 60$^{o}$C in 0.5 ml BTS-450 (Beckman tissue solubilizer: 0.5N quaternary ammonium hydroxide in toluene).

## Statistical Analysis

Analyses of variance were run using a fully nested design with breed as the main effect. Cross interactions were considered within each breed in the following manner:

Sex within each breed

Age within each sex

Thoracic x celiac within each age

No treatment x Estrogen treatment within each tissue site

Significant differences between total and free activities were determined by the Student's t Test for paired samples.

## IV. RESULTS

### Protein Yield of Subcellular Fractions

The major and most consistent differences in the protein yields of the "lysosomal" and microsomal fractions were seen as a function of age within sex and breed at each site. (Tables 1-6)

The "lysosomal" protein yield in the SR (both sexes) did not vary significantly with age at either tissue site. However, in young WC the protein yield of this fraction was high and dropped as the birds matured. The yield in WC males was highest at 6 weeks at both tissue sites. In the celiac segment of the WC female, protein yield was also highest at 6 weeks but was maximal in the thoracic segment at 6 months. (Tables 1 and 2; Figures 2a and 2b)

Generally the microsomal protein yield increased with age with the exception of the yield in the WC female, which was maximal at 6 weeks and was significantly lower than in other birds in the celiac segment at 6 years of age. (Tables 1, 2, 4; Figures 2c, 2d)

No significant differences between aortic sites in "lysosomal" and microsomal protein yields were seen in either breed. (Tables 1 and 3)

Only a few scattered differences in the protein yields of fractions between breeds or sexes were observed and no trends are apparent. (Tables 1, 4, and 5)

Estrogen incubation significantly increased the "lysosomal" protein yield in both the thoracic and celiac segments of 6 week

old SR male pigeons, eliminating breed differences. (Tables 1, 4 and 6; Figure 3a) The age profiles of the males subsequent to estrogen administration were also similar: maximal at 6 weeks (both tissue sites), minimal at 6 months and then high again at 6 years (celiac only). (Tables 1 and 2; Figures 3a and 3b) Estrogen treatment also caused an increase in "lysosomal" protein yield at 6 weeks in the WC female and at 6 months in the SR female, but only in the celiac segment, so that yields in the celiac became significantly different from those in the thoracic segment and between breeds for the female. (Tables 1, 3, 4 and 6) The "lysosomal" protein yield in the SR male celiac segment was significantly higher than any of the other birds at 6 years. Estrogen treatment of the tissue did not alter the yield in the SR, but did increase the yield in the WC so that they were no longer significantly different. Also of note is the particularly low yield of the SR female at 6 weeks (significantly different from other birds only in the celiac segment), which is not altered significantly by estrogen treatment. (Tables 1, 2, 3 and 4; Figures 2a, 2b, and 3a)

Estrogen treatment of tissue increased microsomal protein yield at 6 weeks in the WC male and female thoracic, SR male thoracic and celiac (at 6 months also) enhancing age differences. (Table 2) The SR female tissue was not responsive. Notably the WC female celiac showed a significant drop at 6 weeks subsequent to estrogen treatment, which was paralleled by the large increase in the lysosomal yield at 6 weeks. (Figure 3a)

Figure 2a. Protein Yields of the Lysosomal Fraction from the Upper Thoracic Aortas of White Carneau (WC) and Show Racer (SR) Pigeons.

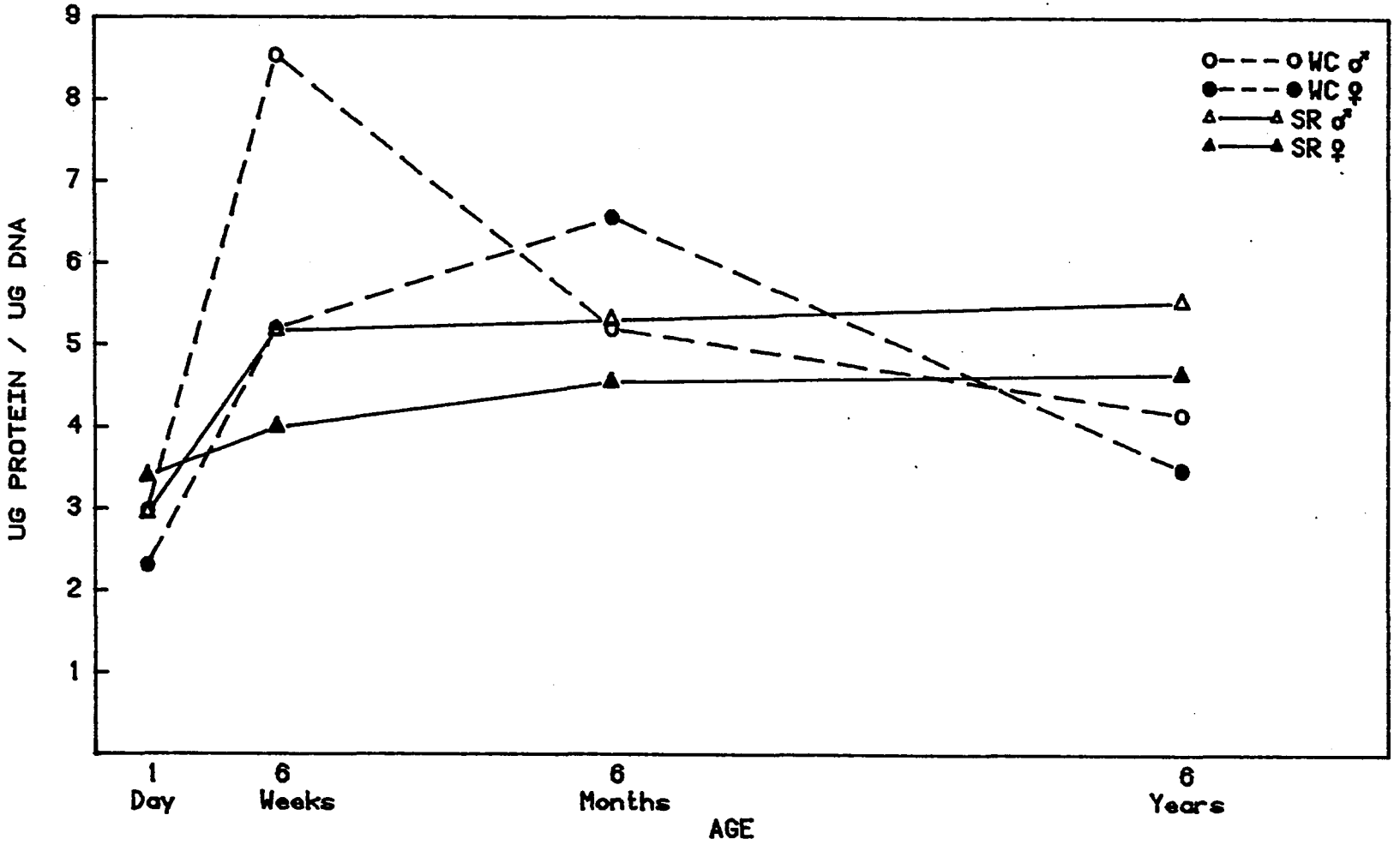


Figure 2b. Protein Yields of the Lysosomal Fraction from the Celiac Bifurcation of White Carneau (WC) and Show Racer (SR) Pigeons.

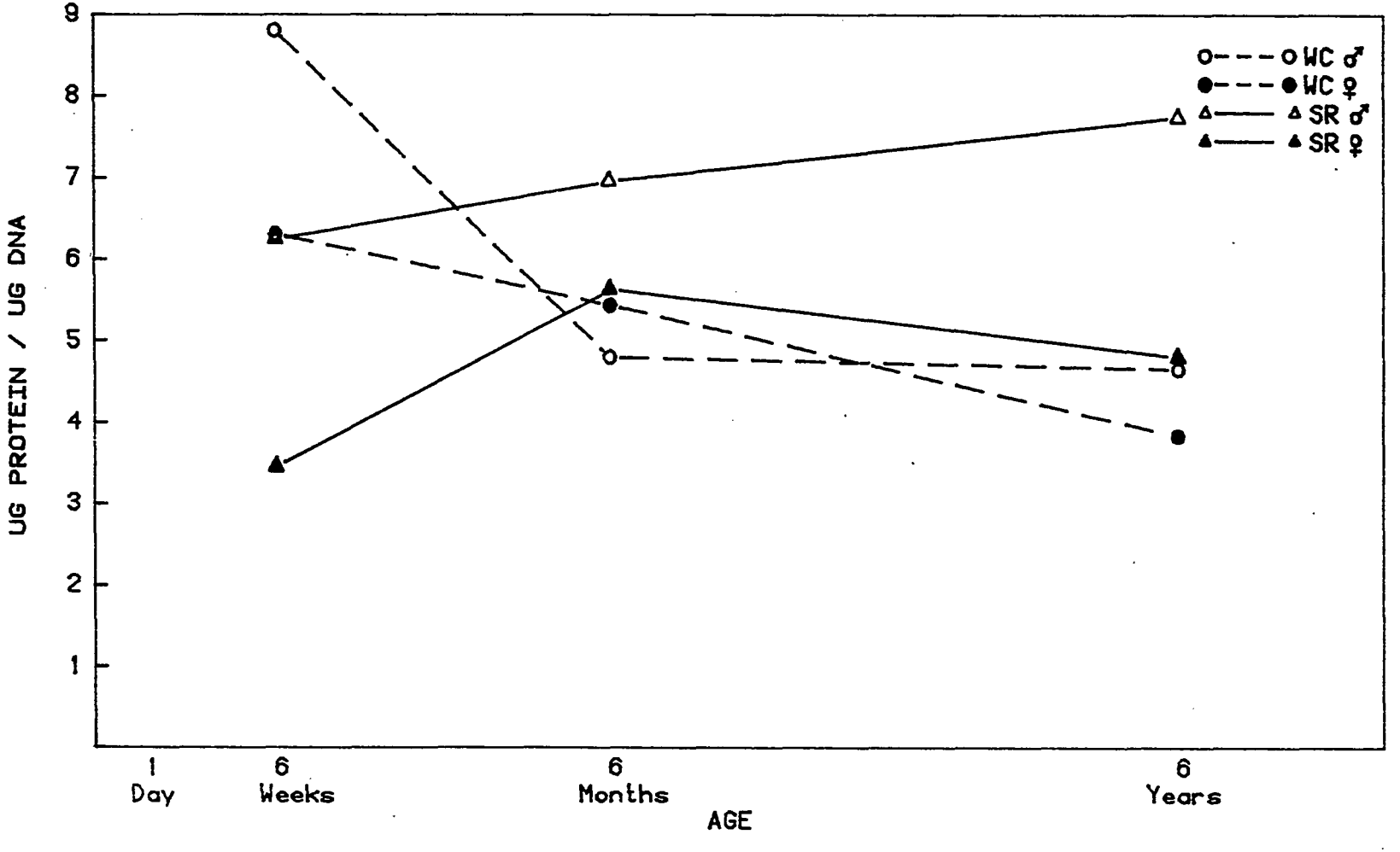


Figure 2c. Protein Yields of the Microsomal Fraction from the Upper Thoracic Aortas of White Carneau (WC) and Show Racer (SR) Pigeons.

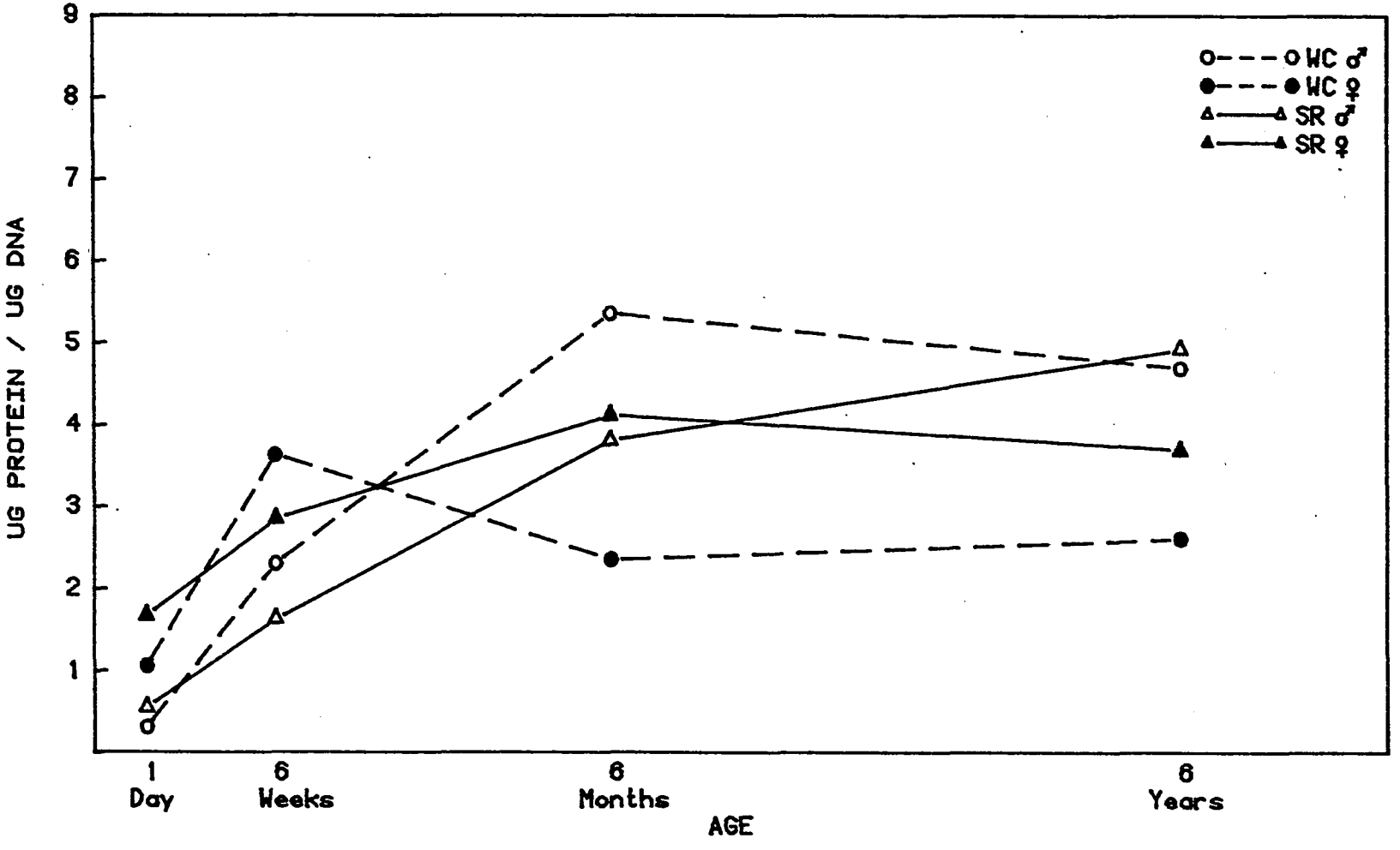


Figure 2d. Protein Yields of the Microsomal Fraction from the Celiac Bifurcation of White Carneau (WC) and Show Racer (SR) Pigeons.

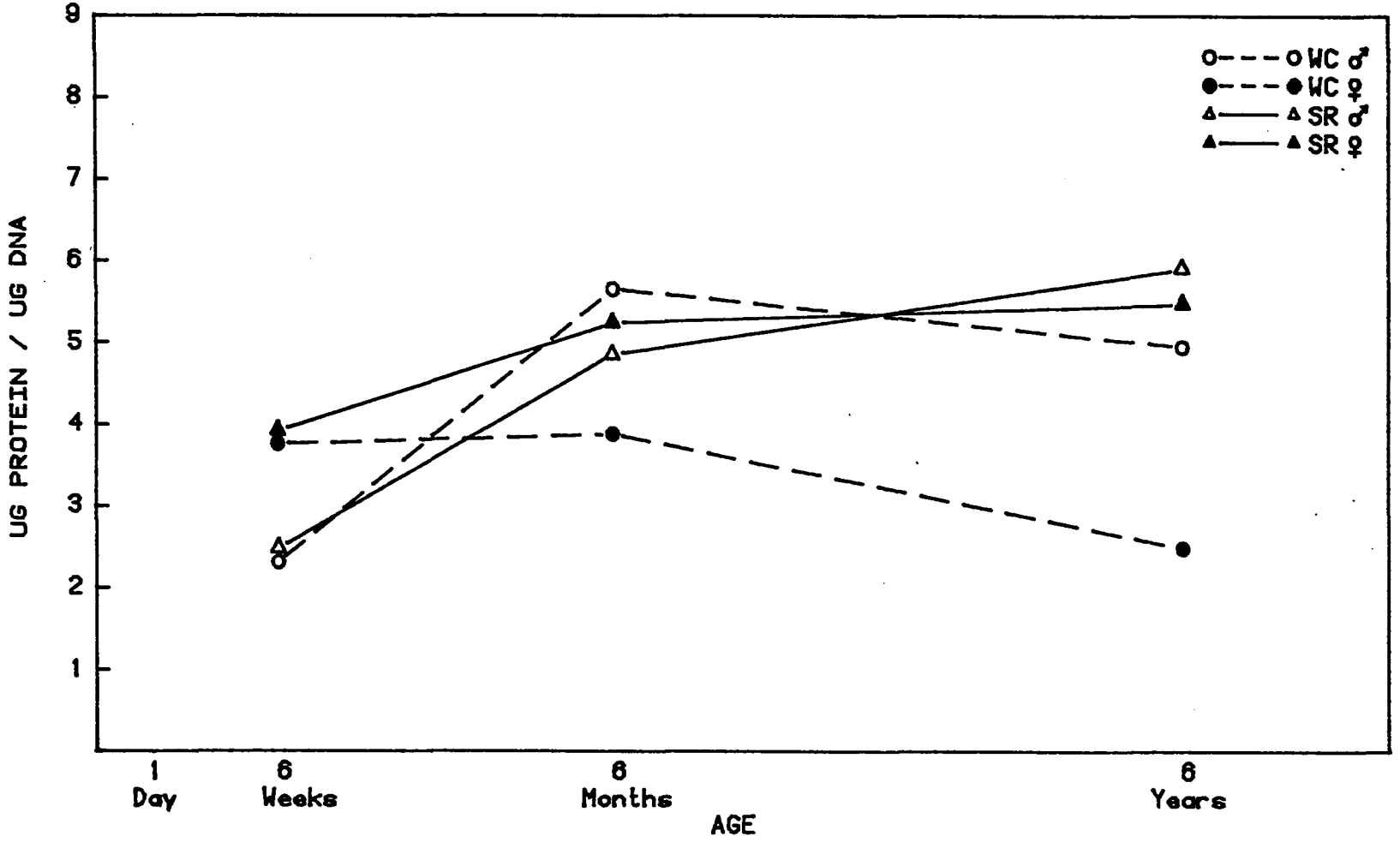
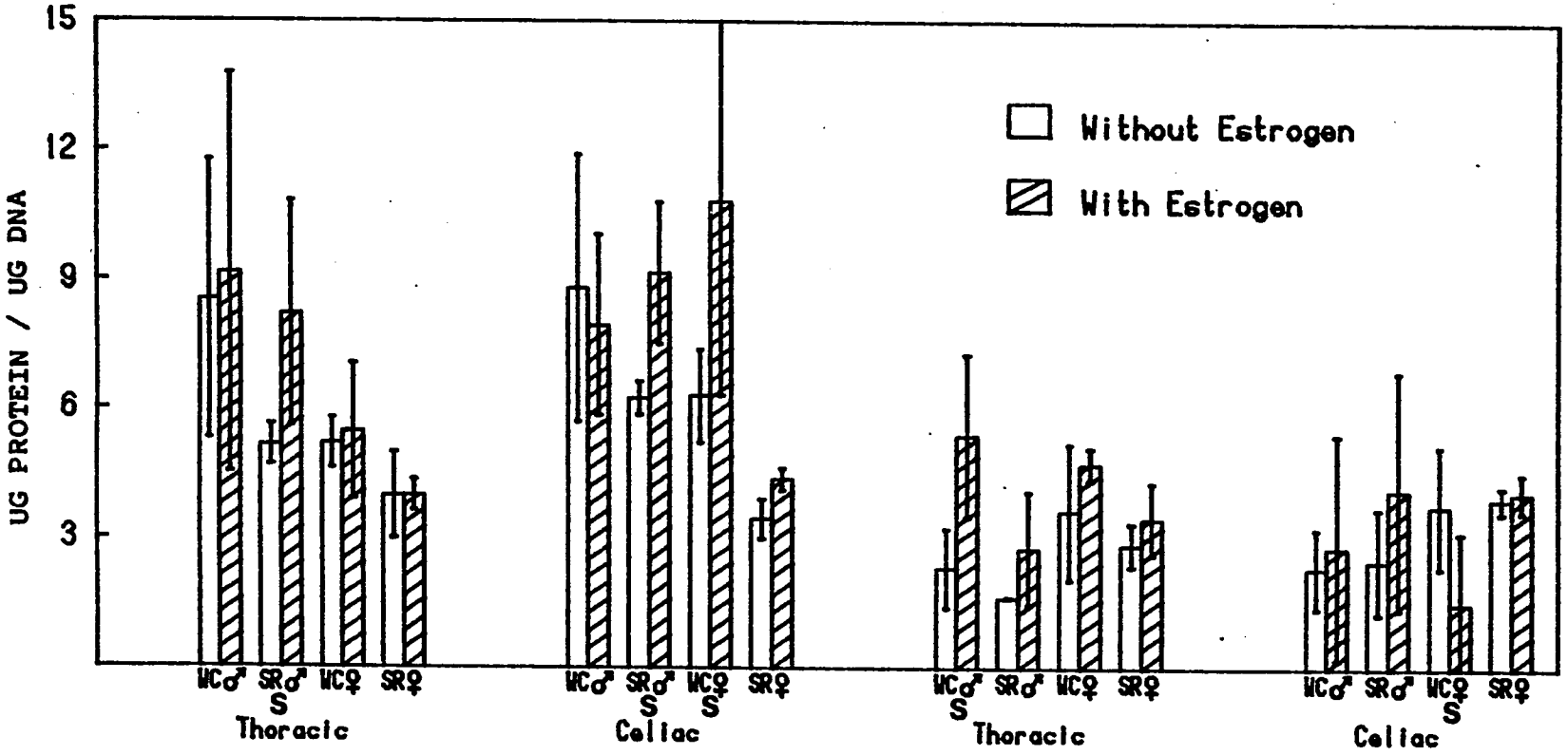


**Figure 3a**

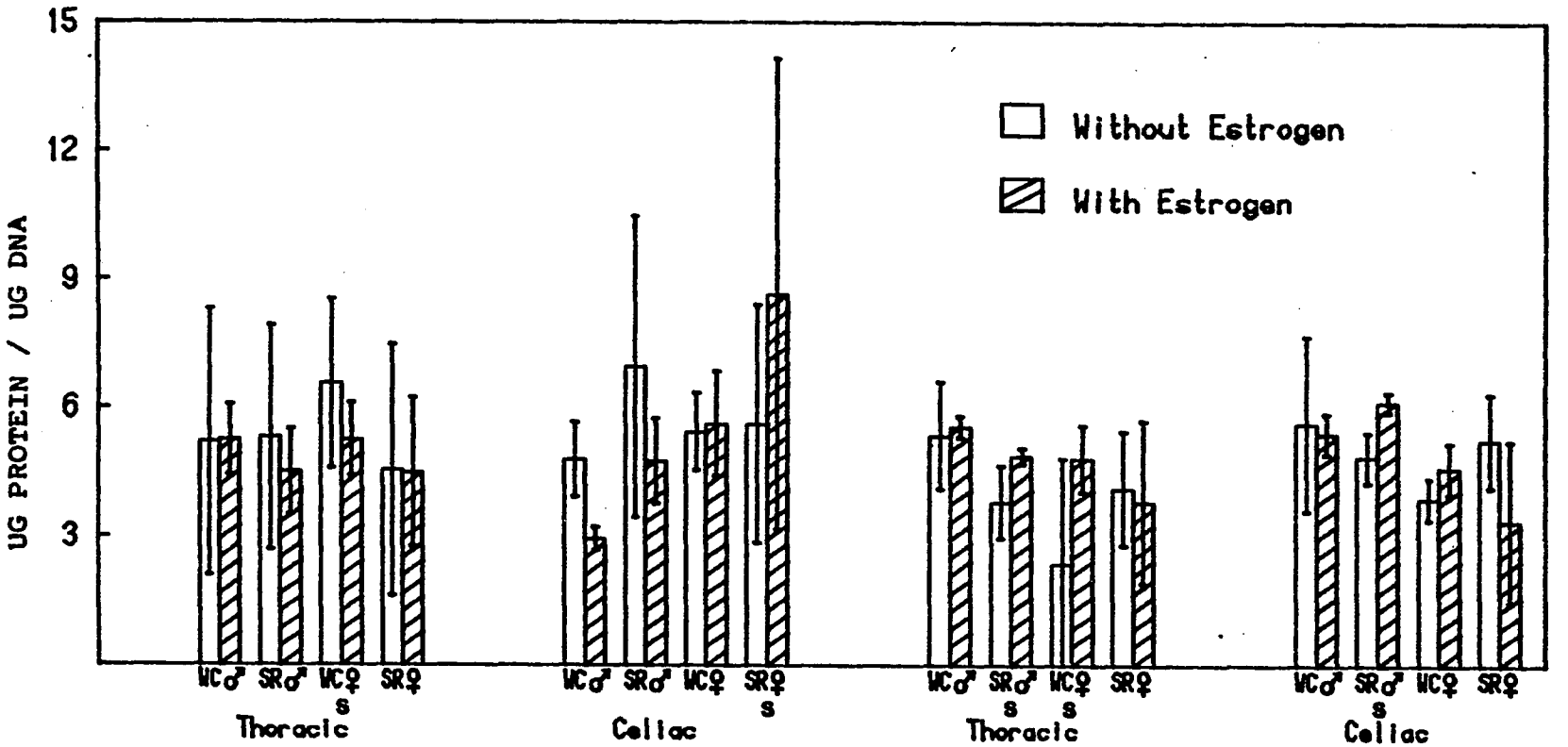
Protein Yield of the "Lysosomal" and Microsomal Fractions from the Upper Thoracic and Celiac Bifurcation of Six Week Old White Carneau (WC) and Show Racer (SR) Aortas.




S=significant difference ($P<0.05$) between treatments; statistical analysis was done by the Student's Test for paired samples.
Error bars represent ± 1 SD. WC=White Carneau; SR=Show Racer

**Figure 3b**

Protein Yield of the "Lysosomal" and Microsomal Fractions from the Upper Thoracic and Celiac Bifurcation of Six Month Old White Carneau (WC) and Show Racer (SR) Aortas.




s = significant difference ($P<0.05$) between treatments; statistical analysis was done done by the Student's Test for paired samples.
Error bars represent ± 1 SD. WC=White Carneau; SR=Show Racer

## Enzyme Activities

### A. N-Acetyl-B-Hexosaminidase (NAHase) Activity

Since no significant sex differences were found in NAHase activity except when estrogen treated, the data for both sexes was pooled for presentation in Tables 7 and 8. Figures 5 a-c show data from males and females where the effect of estrogen was significantly different between sexes.

#### 1. Age and breed differences in the thoracic aorta.

Both Brij-35 and free NAHase activities in the homogenate and all fractions decreased with age in each breed. However, activity in the SR was 71% lower at 6 weeks than at 1 day and did not change significantly from 6 weeks to 6 years. The activity in the WC was 28% lower at 6 weeks than at 1 day and 64% lower at 6 months. (Table 9; Figures 4a, b, c)

The relative subcellular distribution of NAHase activity was similar in both breeds. The "lysosomal" fraction contained 40% of the total activity, the soluble fraction, 40-50%, and the microsomal fraction contained only a negligible amount of NAHase activity.

The only significant difference in NAHase activity in the upper thoracic segment between breeds occurred at 1 day where the SR NAHase activity was higher than the WC in all subcellular fractions. (Tables 7 and 10)

#### 2. Age and breed differences in the celiac bifurcation.

The total NAHase activity in both breeds was lower at 6 months

than at 6 weeks, but this decrease was statistically significant only in the WC. The total activity in the WC was significantly higher than in the SR at 6 weeks, similar to the activity in the SR at 6 months, and higher than in the SR at 6 years. (Tables 8 and 10; Figure 4a) Upon fractionation of the homogenate, at 6 weeks the free lysosomal activity and Brij-35 soluble activity in the WC was significantly higher than in the SR. (Table 10; Figures 4b and 4c) The higher free activity without higher Brij-35 activity is indicative of differences in latent activity which will be presented in a later section. At 6 years the only breed difference occurred in the soluble fraction where activity in the WC was higher than in the SR. (Table 10; Figure 4c)

The relative subcellular distribution of NAHase activity in the celiac segment was the same as seen for the upper thoracic aorta at 6 weeks and 6 months. However, at 6 years as much as 65% of the NAHase activity in the WC was soluble, whereas only 44% was soluble in the SR.

The only significant difference between tissue sites in NAHase activity occurred in the 6 year old WC where the soluble activity in the celiac segment was higher than in the thoracic segment. (Tables 7 and 8; Figure 4c)

3. The effect of 17B-estradiol on NAHase activity.

Only a few scattered changes in age, breed, or aortic site differences occurred subsequent to estrogen treatment (Tables 7,8,10, and 11), but no meaningful trends were observed.

Subsequent to incubation of the thoracic aorta with 17B-estradiol, free NAHase activity in the soluble fraction significantly increased in the SR male, but decreased in the WC male. The Brij-35 NAHase activity in this fraction from SR female aortas significantly decreased subsequent to estrogen incubation. The NAHase activity in the SR female also decreased in the "lysosomal" fraction and increased in the microsomal fraction. (Figure 5a) The activity in the WC female homogenate increased, and this increase was seen in the "lysosomal" and microsomal (statistically significant) fractions.

Statistically significant effects of estrogen treatment at other ages are few and scattered without any meaningful trends.

4. The effect of Brij-35 on N-acetyl-B-hexosaminidase activities in the soluble fraction.

Addition of Brij-35 to the assay medium caused a significant decrease in NAHase activity in the soluble fraction (Tables 7, 8 and 12). In some cases estrogen treatment appeared to enhance inhibition by Brij-35 (Tables 7, 8 and 12). Only a few scattered differences and no major trends in the decreased NAHase activity were observed (Tables 13 and 14).

5. Latent NAHase Activity

The latent activity was extremely variable; however, a few significant differences were seen. The percents of the Brij-35 activity released and the actual levels of activity released

by Brij-35 in the "lysosomal" fraction are presented in Table 15. In both breeds the actual amount of enzyme activity released decreased with age, however in WC the total activity decreased to a greater degree so that the percent latent activity actually increased with age. In the SR no changes in the percent latency were seen with age (Table 16).

The only significant difference in latency between breeds was seen in the celiac bifurcation at six weeks of age (Table 17). At this age the WC did not show any significant latent enzyme activity whereas 33% of the total activity in the "lysosomal" fraction of the SR was latent.

The only significant effect of estrogen on the enzyme latency was seen in the celiac bifurcation of six month old SR (Table 15): estrogen completely eliminated all latent activity. At 6 years estrogen decreased the latency in the celiac bifurcation of both breeds, but to a greater extent in the SR so that a breed difference appeared. Estrogen treatment also decreased latency in the WC at 6 weeks in the thoracic (Tables 15 and 17).

## B. Acid Phosphatase (APase) Activity

### 1. Sex, age and breed differences in the upper thoracic aorta.

Unlike NAHase, APase activity was sex dependent. In general, statistically significant sex differences were seen as early as 1 day in the SR, but not until 6 years in the WC (Figures 6a-d; Tables 18 and 22). Generally, estrogen treatment amplified sex differences

in both breeds by increasing the male and/or decreasing the female activities, so that a greater number of statistically significant sex differences were seen (Table 19 and 22). However, at 6 years estrogen treatment eliminated sex differences seen in the WC with no treatment.

The SR (both sexes) total activity was significantly lower at 6 weeks than at 1 day, whereas the WC activity tended to be higher at 6 weeks than at 1 day, but only significantly higher in the WC male (Tables 18 and 23; Figure 6a). The activity in the WC male reached a maximum at 6 months and was lower at 6 years. The total activities in the SR (both sexes) and WC female did not change significantly from 6 weeks to 6 years.

The majority of the APase activity was found in the "lysosomal" fraction, about 50% at 1 day increasing to 65% by 6 years. The lysosomal activity followed the same age trend as the homogenate activity (Figures 6b and 6c; Tables 18 and 23). However, the soluble activity decreased with age in both breeds and sexes from 1 day to 6 years (Tables 18 and 23; Figure 6d). At 1 day the soluble activity in the SR male was significantly higher than in the WC (both sexes) or in the SR female. The microsomal activity was lower in the SR (both sexes) at 6 weeks than at 1 day, but did not vary significantly from 6 weeks to 6 years. The microsomal fraction in the thoracic aortas of WC (both sexes) did not change significantly from 1 day to 6 years (Figure 6e). No major changes in age trends were seen subsequent to estrogen treatment (Table 24).

The major breed differences occurred at 1 day in the male pigeons

such that the activity in the SR male was significantly higher than in the WC male in all subcellular fractions (Table 18 and 25; Figures 6a-6e).

Estrogen treatment of tissue increased the APase activity in the SR male at 1 day and 6 weeks, but decreased it at 6 months. In the WC male estrogen treatment increased the activity at 1 day and 6 years, decreased it at 6 weeks, and had no effect at 6 months. Because the male pigeons react differently at the same ages, significant breed differences at 6 weeks (SR total activity is higher) and at 6 months (SR total and lysosomal activities are lower) appear subsequent to estrogen treatment (Tables 19, 24 and 26). The microsomal activity in the SR female increased at 1 day after estrogen treatment so that it became significantly higher than in the WC female.

2. The affect of 17B-estradiol treatment on APase activity in the thoracic aorta

In one day aortas the total APase activity tended to increase subsequent to incubation with estrogen in both sexes of both breeds, but only in the WC was the increase significant. However, APase activity did increase significantly in the soluble fraction of the SR male and in the microsomal fraction of the SR female (Figure 8a; Table 19). The WC male also showed a significant increase in APase activity in the microsomal fraction, making the activity in this fraction similar to the activity in the SR male and WC female. The SR female microsomal activity subsequent to estrogen incubation

was at least 1.7 fold higher than the SR male or the WC of either sex.

In 6 week aortas only the activity in the SR male homogenate increased significantly subsequent to estrogen treatment (Table 19; Figure 8b). No notable changes occurred at 6 months in either sex of either breed. At 6 years the activity in the WC homogenate increased subsequent to estrogen incubation (male $P < 0.05$; female $P < 0.1$).

### 3. Sex, age, and breed differences in APase activity in the celiac bifurcation

Sex differences were seen at 6 weeks in the SR and at 6 months (earlier in the thoracic) in the WC. At 6 years sex differences occurred in the "lysosomal" fraction of the SR and in the microsomal and soluble fraction of the WC (Tables 19 and 27; Figures 7b-e).

As was seen in the thoracic segment, the APase activity in the SR (both sexes) at the celiac bifurcation did not change significantly from 6 weeks to 6 years in any subcellular fraction. However, the total activity in the WC (both sexes) was significantly higher at 6 years than at 6 months. The activity in the WC female was lower at 6 months than at 6 weeks or 6 years in all fractions, and significantly so in the "lysosomal" and soluble fractions (Tables 20 and 23; Figures 7a-e). The microsomal activity from the WC male was higher at 6 months and 6 years than at 6 weeks (Figure 7e).

The major breed differences occurred at 6 weeks and 6 years.

At 6 weeks APase activity was higher in the "lysosomal" fraction of the corresponding sexes in the WC than in the SR (Tables 14 and 28; Figures 7b and 7c). APase activity in the WC male was lower than the SR male in the microsomal but higher than the SR male in the soluble fraction (Tables 20 and 28; Figures 7d and 7e). At 6 years the activity in the WC homogenate (both sexes) was higher than in the SR only in the microsomal fraction (this was also seen at 6 months) whereas the activity in the WC female was significantly higher than in the SR in the soluble fraction (Figures 7b-7e; Tables 20 and 28). As seen in the thoracic segment, the majority of the APase activity was found in the "lysosomal" fraction.

4. Differences between the thoracic and celiac segments

APase activity in the homogenate in the WC male celiac foci was significantly higher than in the thoracic segment. This increased activity was located in the "lysosomal" fraction ($P < 0.1$) and the soluble fraction ($P < 0.05$) (Tables 18, 20, and 29). Although the total activity was similar in the thoracic and celiac segments of the SR (both sexes), the celiac segment contained a higher proportion of the activity in the microsomal fraction than the thoracic segment (female: $P < 0.1$; male: $P < 0.05$) (Tables 18 and 20).

At 6 months the microsomal fraction of the celiac segment of the males of both breeds contained a higher proportion of the APase activity than the thoracic segments.

At 6 years the lysosomal activity in the WC (both sexes)

celiac segment was higher than the thoracic segment. Also the microsomal activity in the WC male celiac and the soluble activity in the WC female celiac were higher than the corresponding thoracic segment.

### 5. The effect of 17B-estradiol treatment on the APase activity in the celiac bifurcation

As seen in the 6 week thoracic segment, incubation of the celiac segment with estrogen significantly increased the APase activity in the SR male homogenate. However, in the "lysosomal" fraction APase activity increased in both sexes of each breed, with the SR male showing the greatest increase (Figure 9a).

At 6 weeks estrogen treatment had the greatest effect on the SR male celiac segment (Figure 9a). The increased activity in the SR male at 6 weeks eliminated breed differences (Tables 28 and 30), increased sex differences (Table 21), and altered the age profile (Tables 23 and 24), so activity in the homogenate and "lysosomal" fractions were higher at 6 weeks and 6 years than at 6 months (estrogen decreased the SR male activity at 6 months). The WC male had the same age profile (Tables 21, 23, and 24; Figure 9a). The microsomal activity age profile was similar in the untreated and treated tissue but activity increased in the treated tissue (Tables 21, 23, and 24). In the SR female, APase activity increased at 6 months subsequent to estrogen treatment so that activity was lower at 6 weeks and 6 years than at 6 months in the homogenate and "lysosomal" fraction, a pattern opposite to the WC (both sexes)

and the SR male. In the 6 year old WC female, estrogen incubation increased the microsomal activity to eliminate sex differences and increased the lysosomal activity creating a breed difference. Estrogen treatment also increased activity in the soluble fraction in tissue from 6 year olf WC male and decreased activity in the microsomal fraction (Figure 9b; Tables 21, 27, and 30).

6. Latent APase activity

The latent APase activity was quite variable (Table 32). No differences were seen between the thoracic and celiac segments and only a few breed differences appeared; however, no meaningful trends were evident (Tables 33 and 34).

## Summary of Results

**Differences between corresponding aortic sites in WC and SR pigeons, and between thoracic and celiac sites in WC, which appear related to atherogenesis and/or lesion progression are restated below.**

**1: NAHase activities in the homogenate and all subcellular fractions from both aortic sites in 6 week old WC pigeons were greater than those in 6 week old SR. These differences were especially pronounced between the celiac segments.**

**2: At 6 years of age soluble NAHase activity in the WC celiac site was greater than in the SR celiac and in thoracic areas from both breeds.**

**3: Little, if any, latent lysosomal NAHase activity was apparent**

in WC celiac and thoracic sites at 6 weeks of age in contrast to corresponding sites in the SR.

4: APase activities were higher in male aortas than in female aortas at all ages in SR, but only at 6 months and 6 years in WC. Sex differences in APase activities were more pronounced between celiac sites than thoracic areas.

5: "Lysosomal" protein yields were higher from both aortic sites in 6 week WC than from corresponding sites in SR of the same sex.

6: The most noticeable effects of estrogen treatment occurred at 6 weeks of age and produced a marked increase in lysosomal APase activity from celiac segments in both sexes of each breed.

7: Estrogen treatment also increased the "lysosomal" protein yield from celiac sites in 6 week old WC females, SR males, and SR females.

Figure 4a. N-Acetyl-B-Hexosaminidase Activity in Homogenates of the Upper Thoracic and Celiac Segments of White Carneau (WC) and Show Racer (SR) Pigeons.

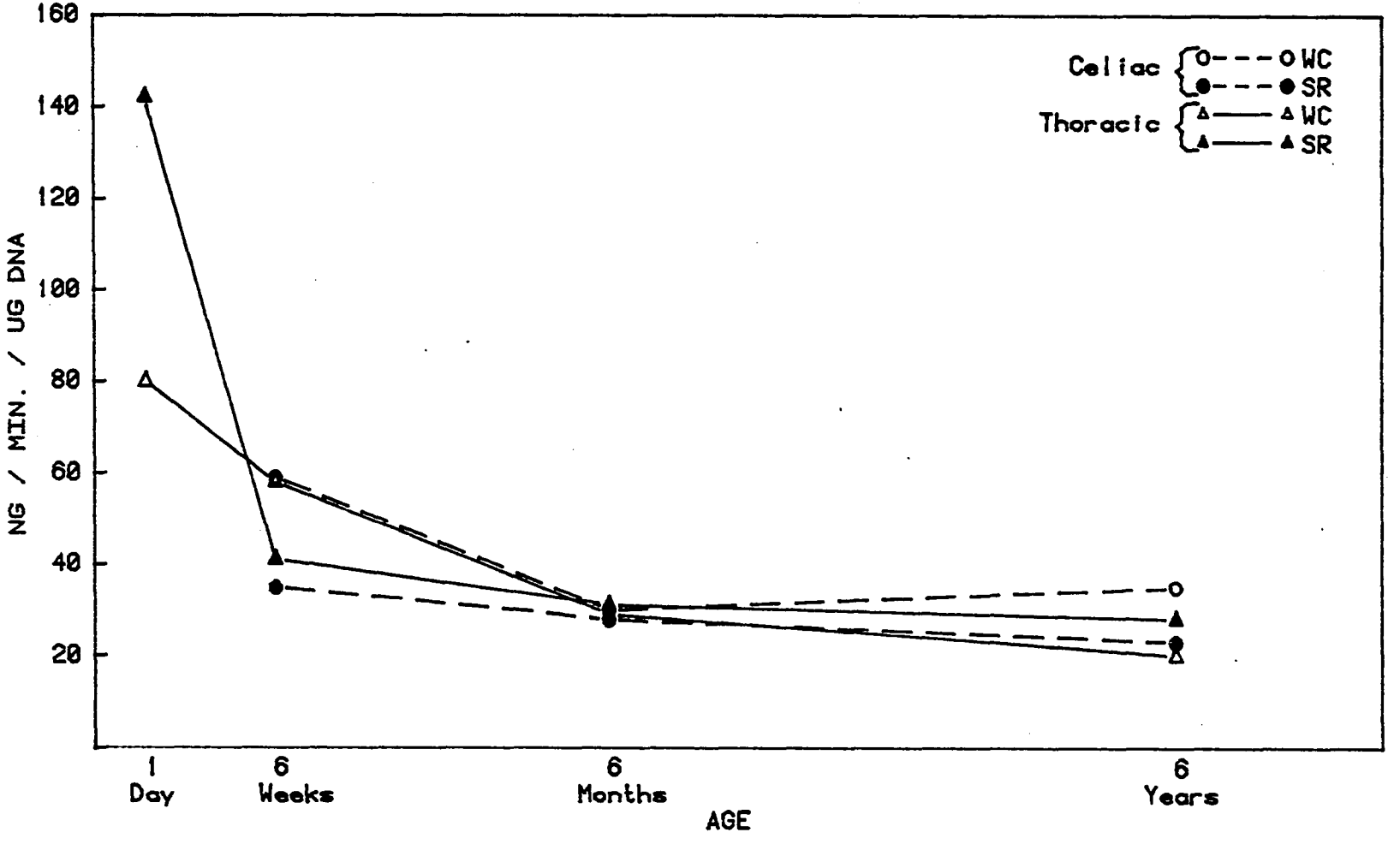


Figure 4b. N-Acetyl-B-Hexosaminidase Activity in the Lysosomal Fraction from the Upper Thoracic and Celiac Segments from White Carneau (WC) and Show Racer (SR) Pigeons.

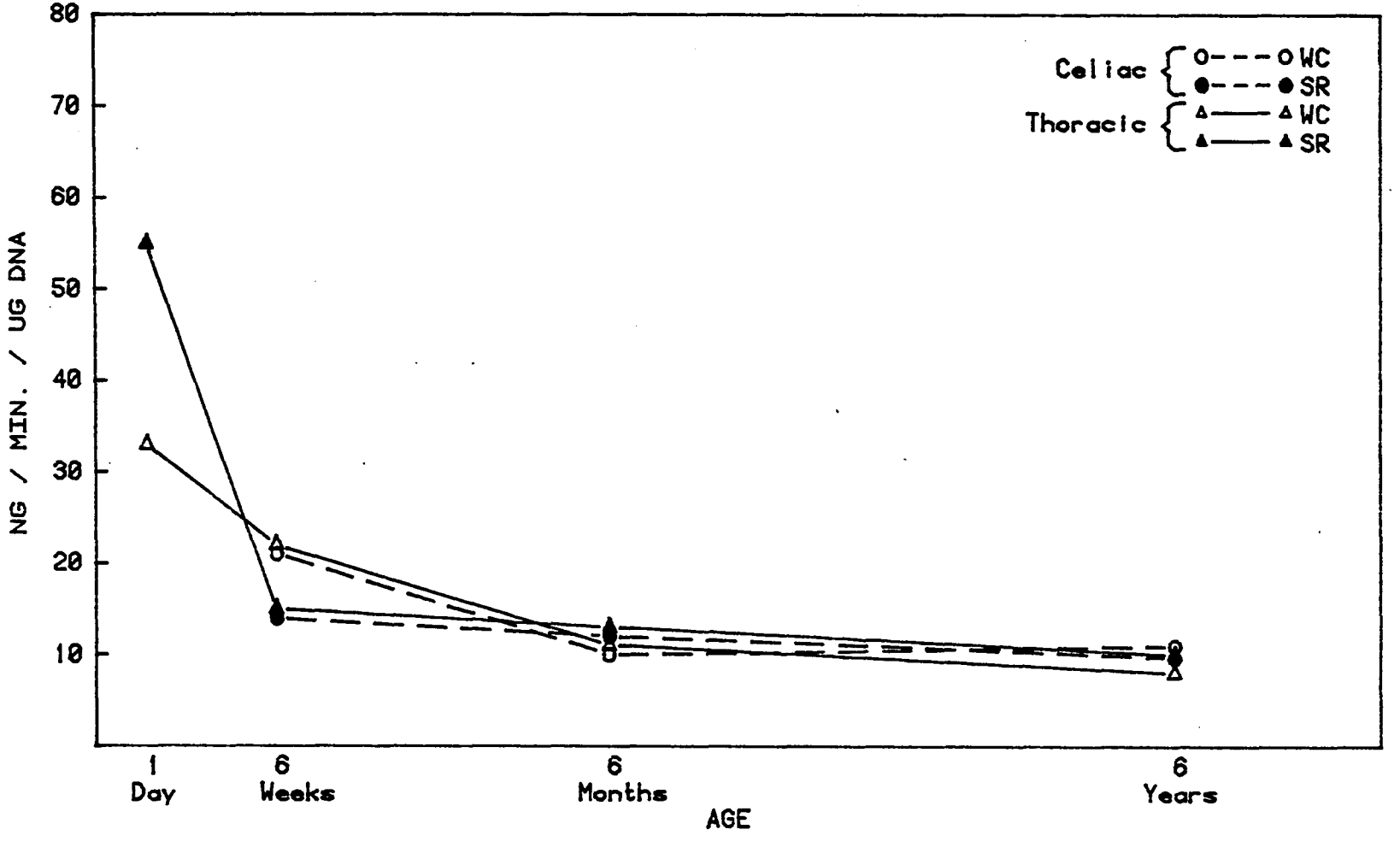


Figure 4c. N-Acetyl-B-Hexosaminidase Activity in the Soluble Fraction from the Upper Thoracic and Celiac Segments from White Carneau (WC) and Show Racer (SR) Pigeons.

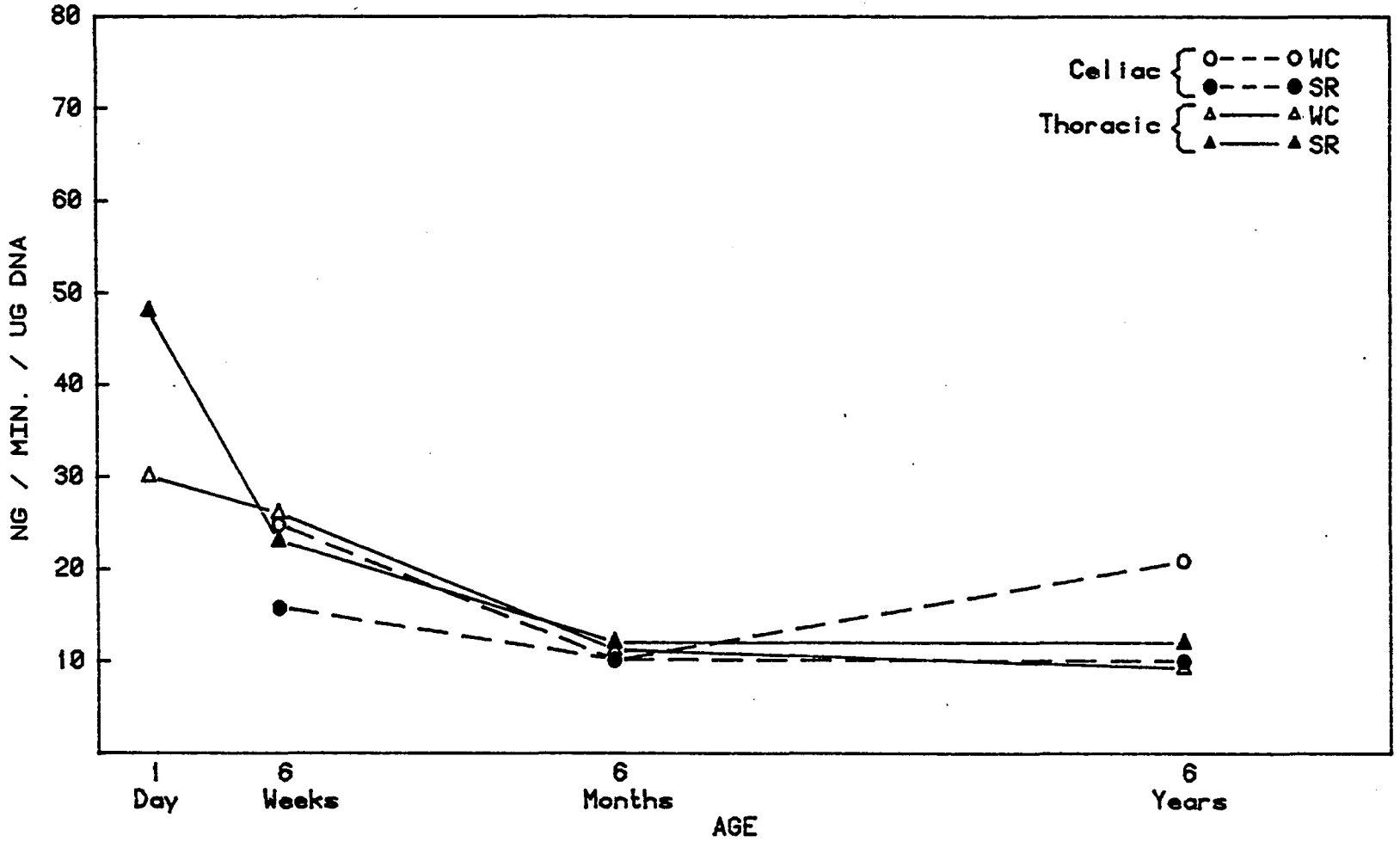
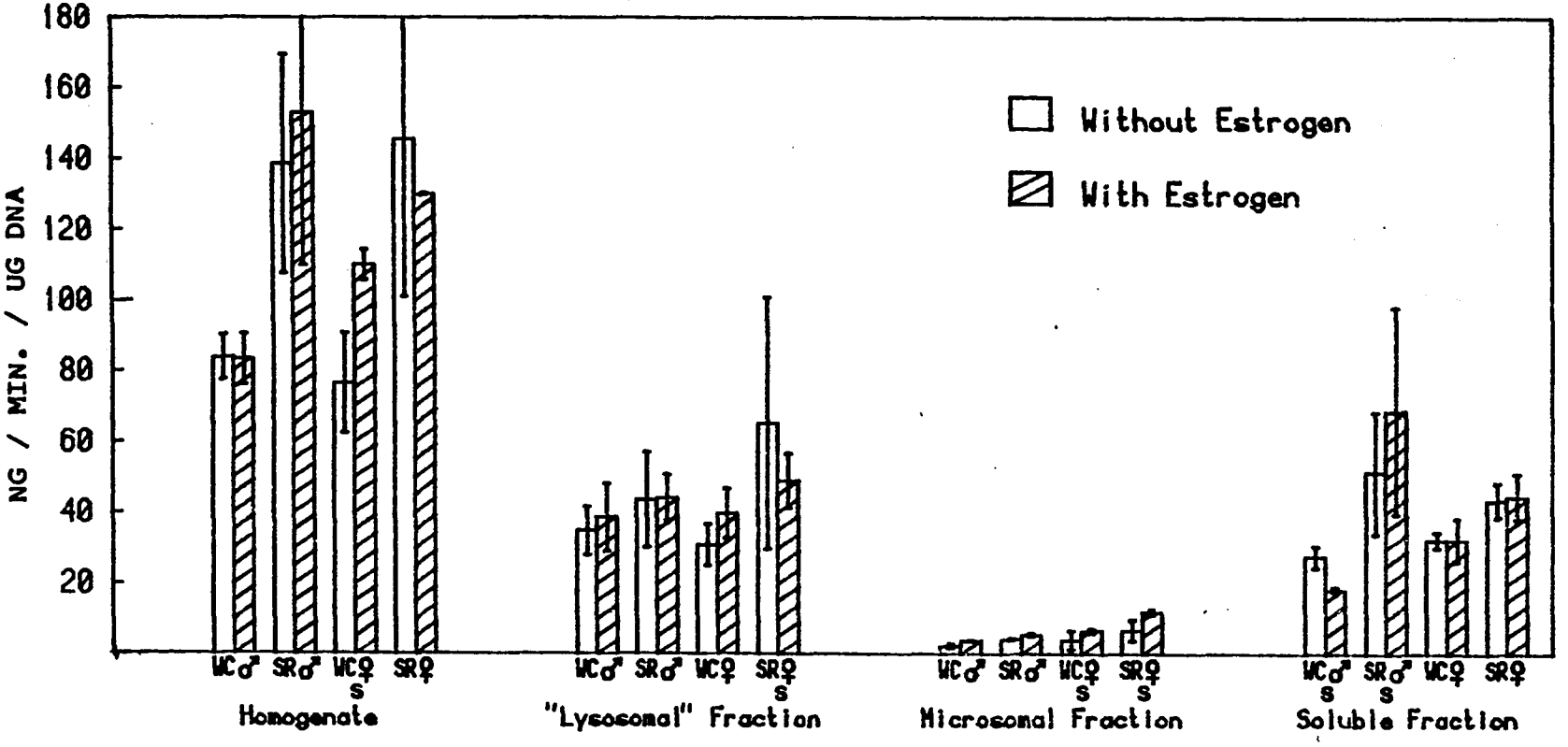


**Figure 5a**

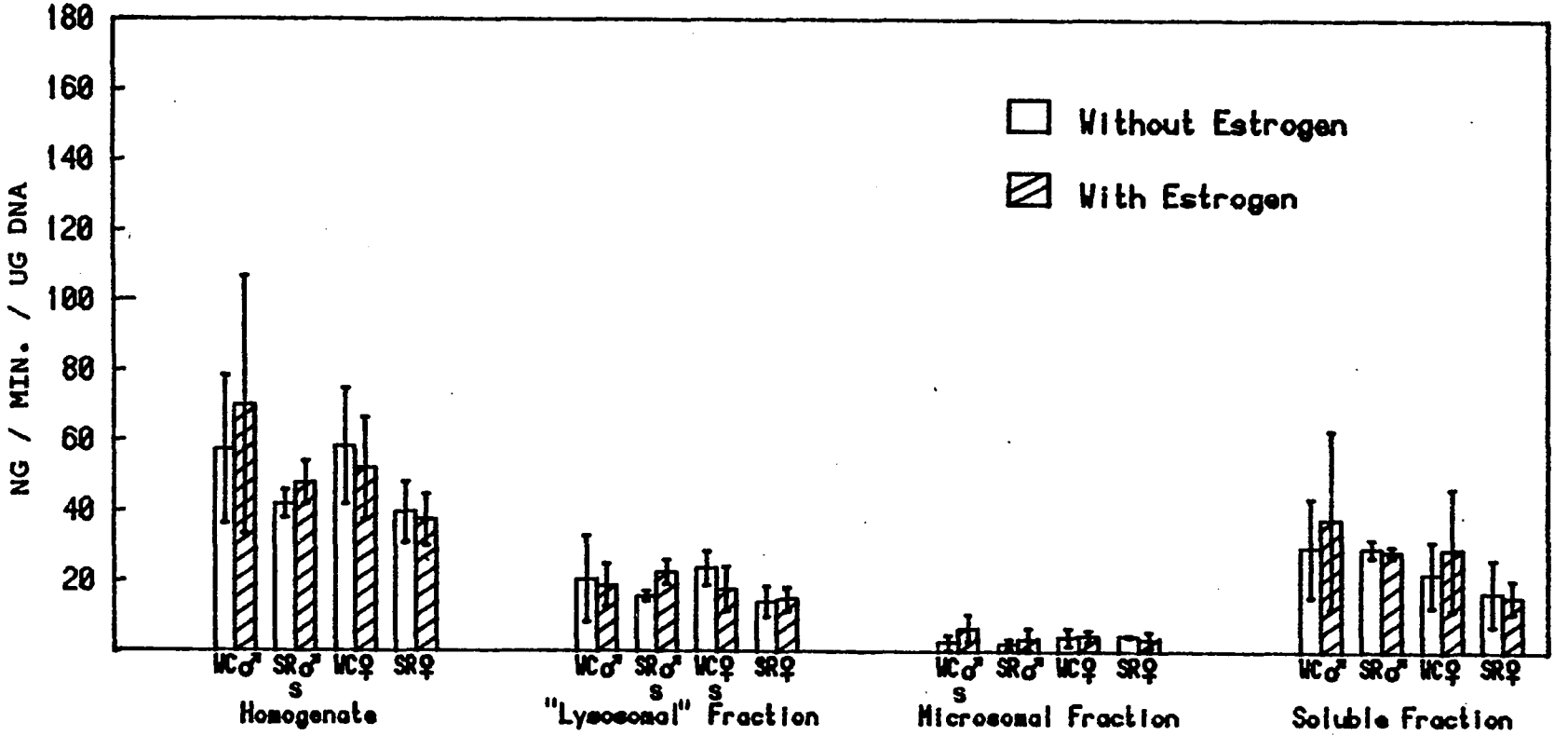
N-Acetyl-B-Hexosaminidase Activity in the Thoracic Aorta of One Day Old White Carneau (WC) and Show Racer (SR) Pigeons with and without Estrogen Treatment.




s = significant difference ($P<0.05$) between treatments; statistical analysis was done by the Student's Test for paired samples.
Error bars represent ± 1 SD. WC=White Carneau; SR=Show Racer

**Figure 5b**

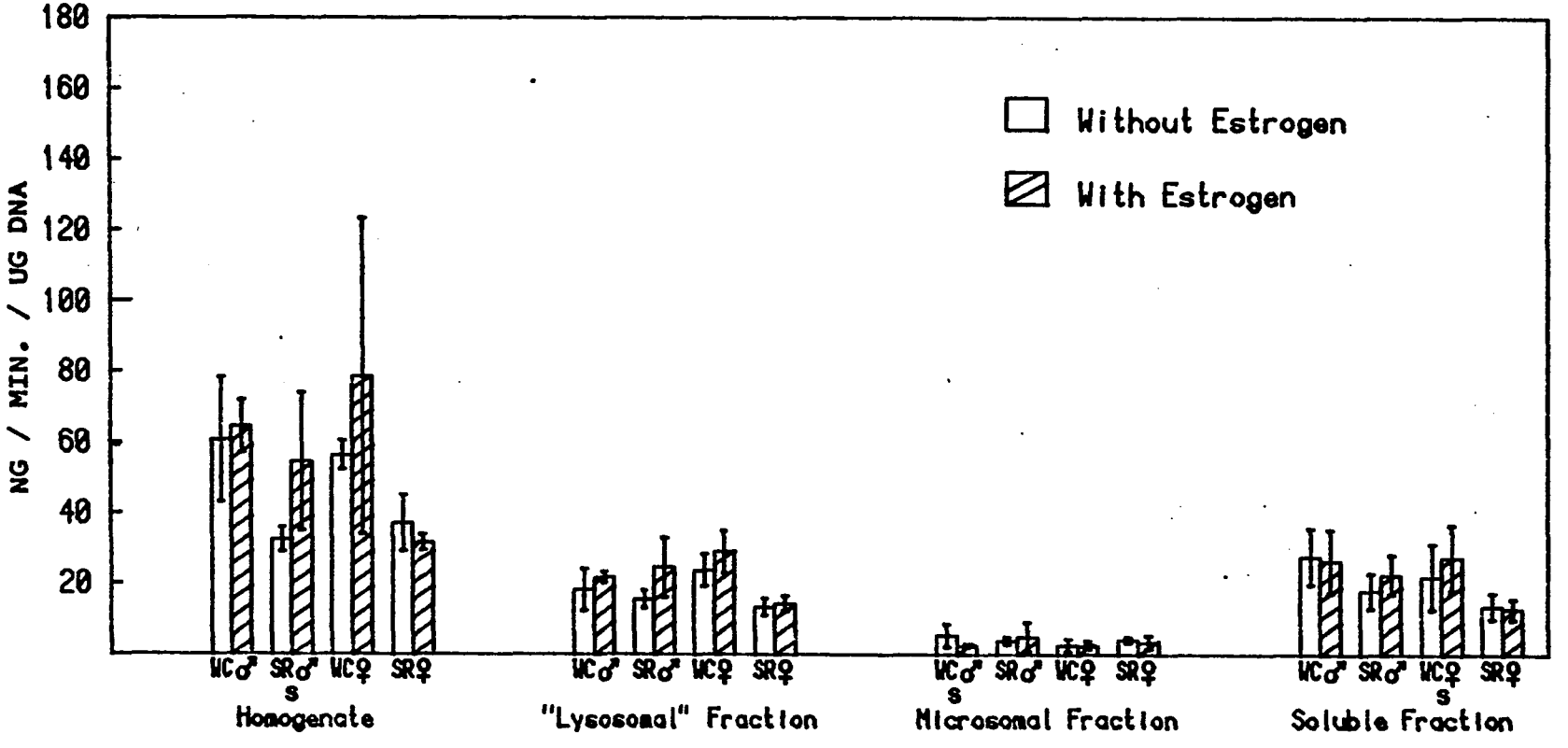
N-Acetyl-B-Hexosaminidase Activity in the Thoracic Aorta of Six Week Old White Carneau (WC) and Show Racer (SR) Pigeons with and without Estrogen Treatment.




s = significant difference ($P<0.05$) between treatments; statistical analysis was done by the Student's Test for paired samples.
Error bars represent ± 1 SD. WC=White Carneau; SR=Show Racer

Figure 5c

N-Acetyl-B-Hexosaminidase Activity in the Celiac Bifurcation of Six Week Old White Carneau (WC) and Show Racer (SR) Pigeons with and without Estrogen Treatment.




s = significant difference ($P<0.05$) between treatments; statistical analysis was done by the Student's Test for paired samples.
Error bars represent ± 1 SD. WC=White Carneau; SR=Show Racer

Figure 6a.

Acid Phosphatase Activity in Homogenates of the Upper Thoracic Aorta from White Carneau (WC) and Show Racer (SR) Pigeons.

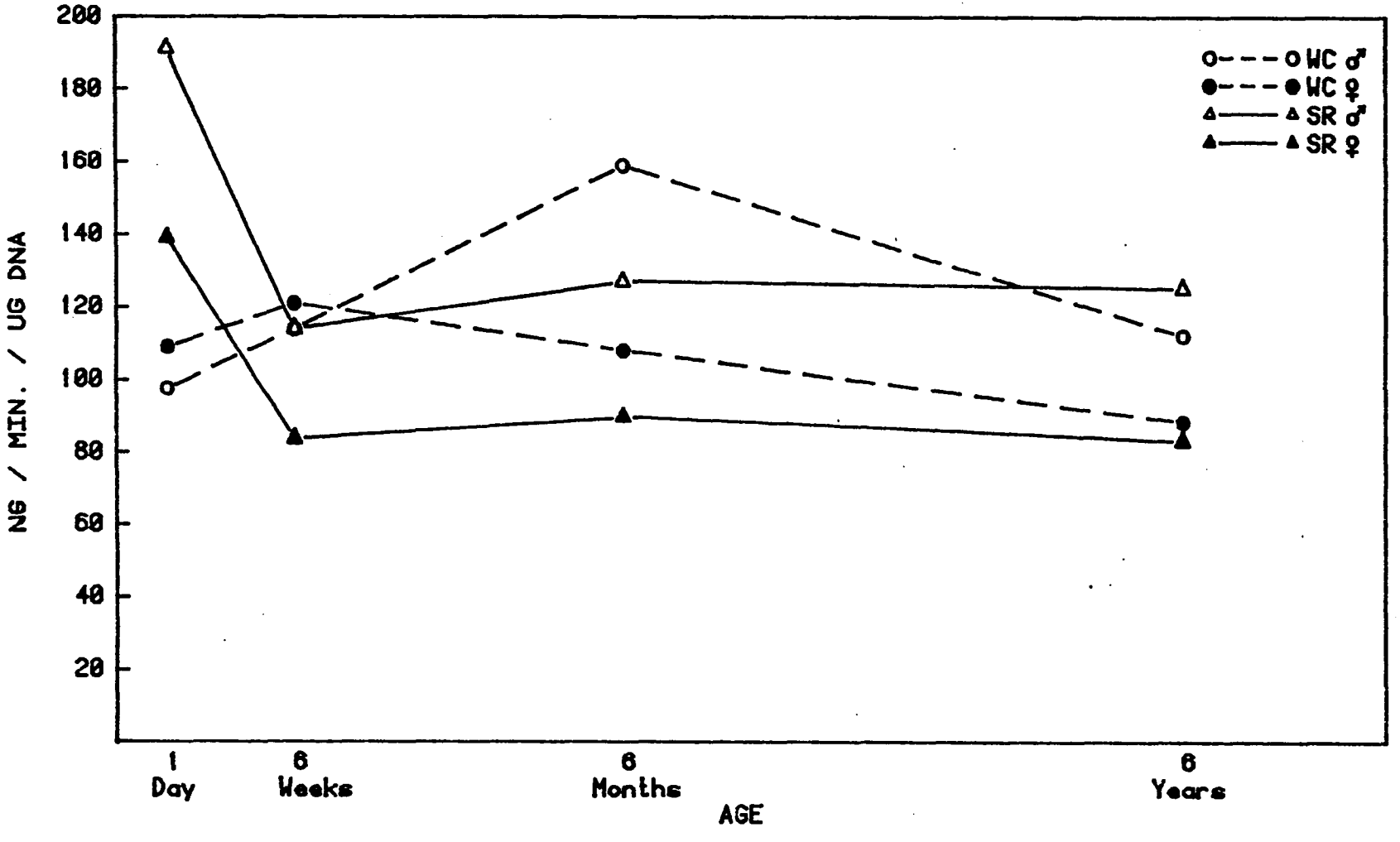


Figure 6b. Acid Phosphatase Activity in the Lysosomal Fraction from the Upper Thoracic Aorta of Female Pigeons.

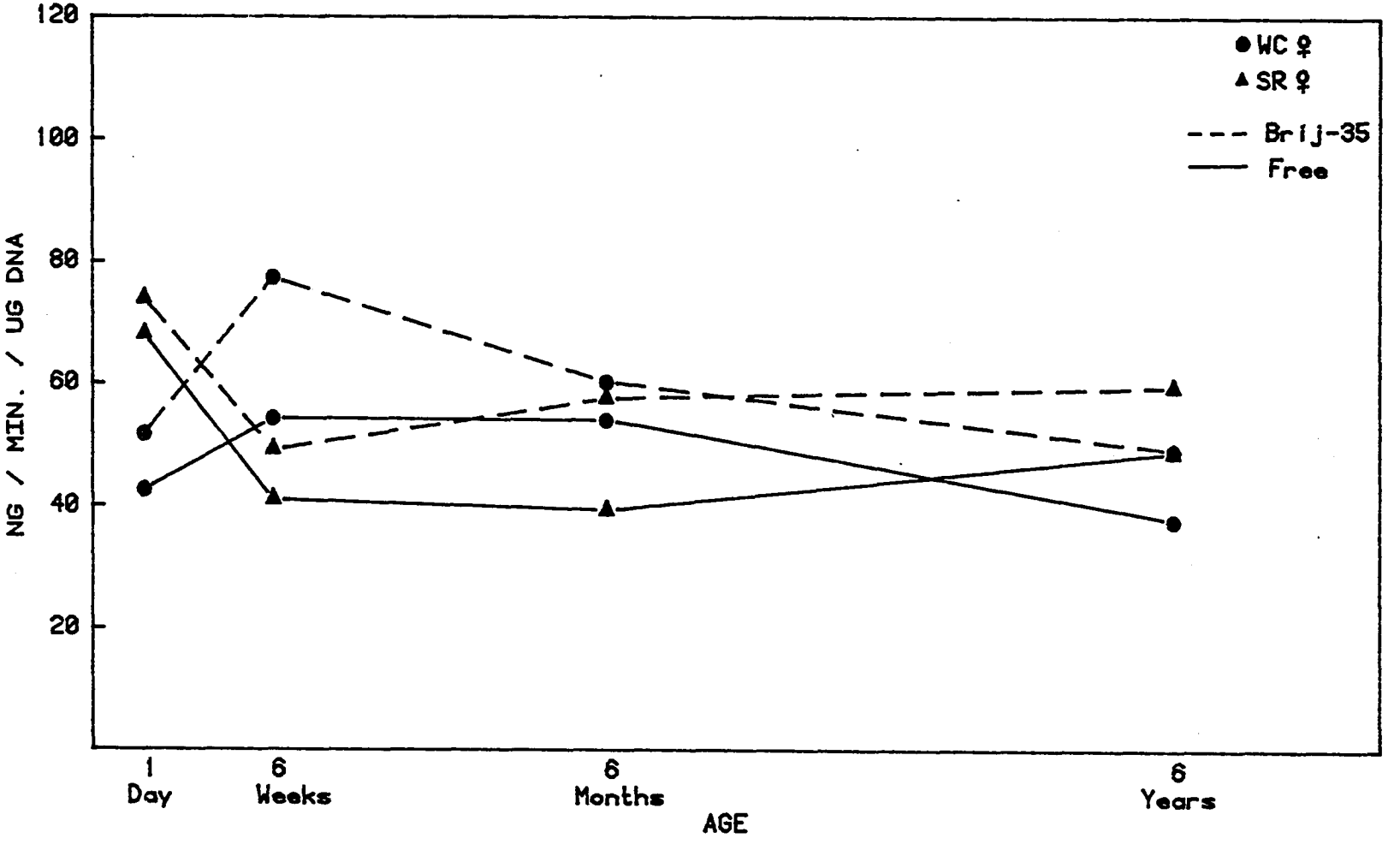


Figure 6c. Acid Phosphatase Activity in the Lysosomal Fraction from the Upper Thoracic Aorta of Male Pigeons.

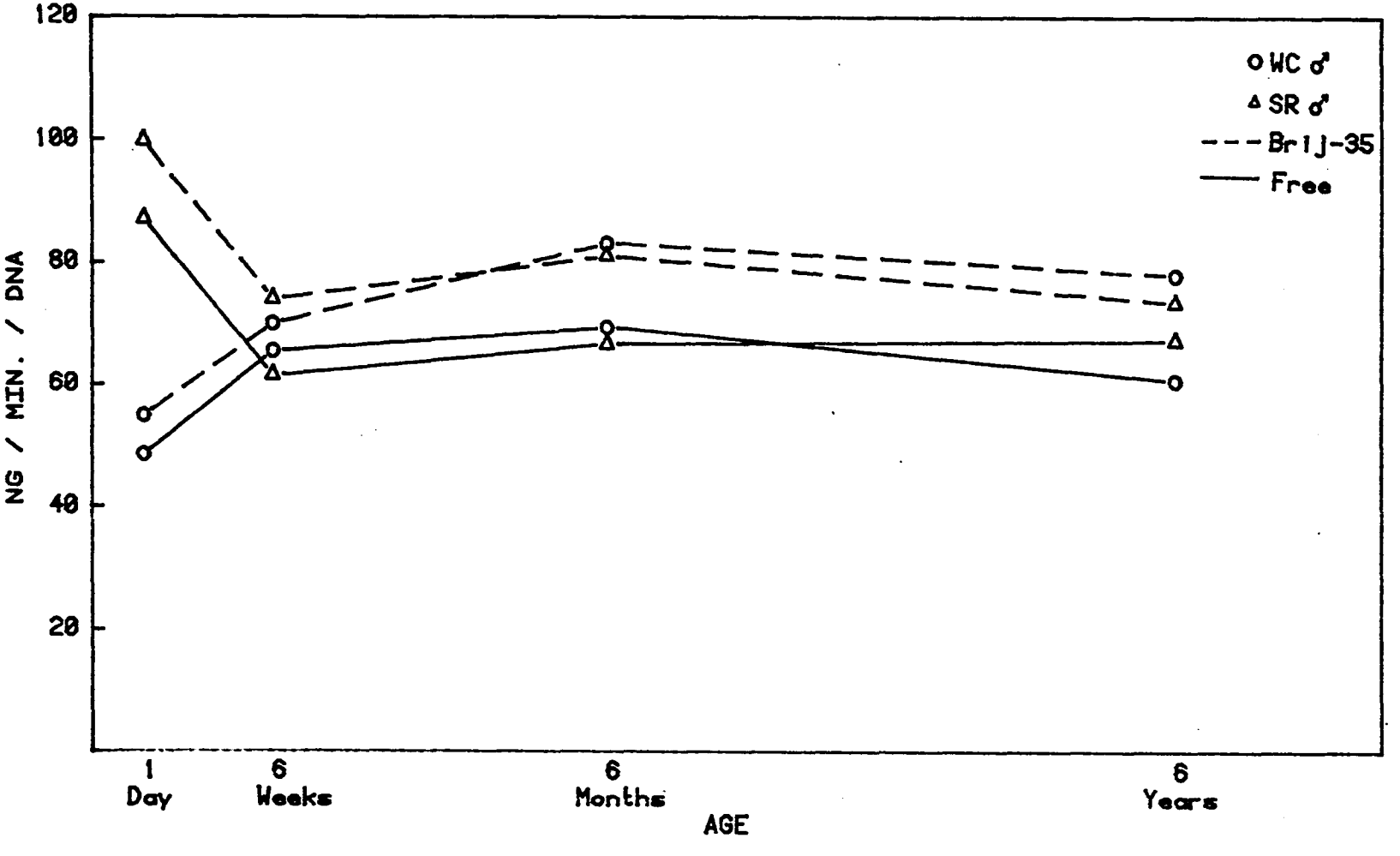


Figure 6d. Acid Phosphatase Activity in the Soluble Fraction from the Upper Thoracic Aorta of White Carneau (WC) and Show Racer (SR) Pigeons.

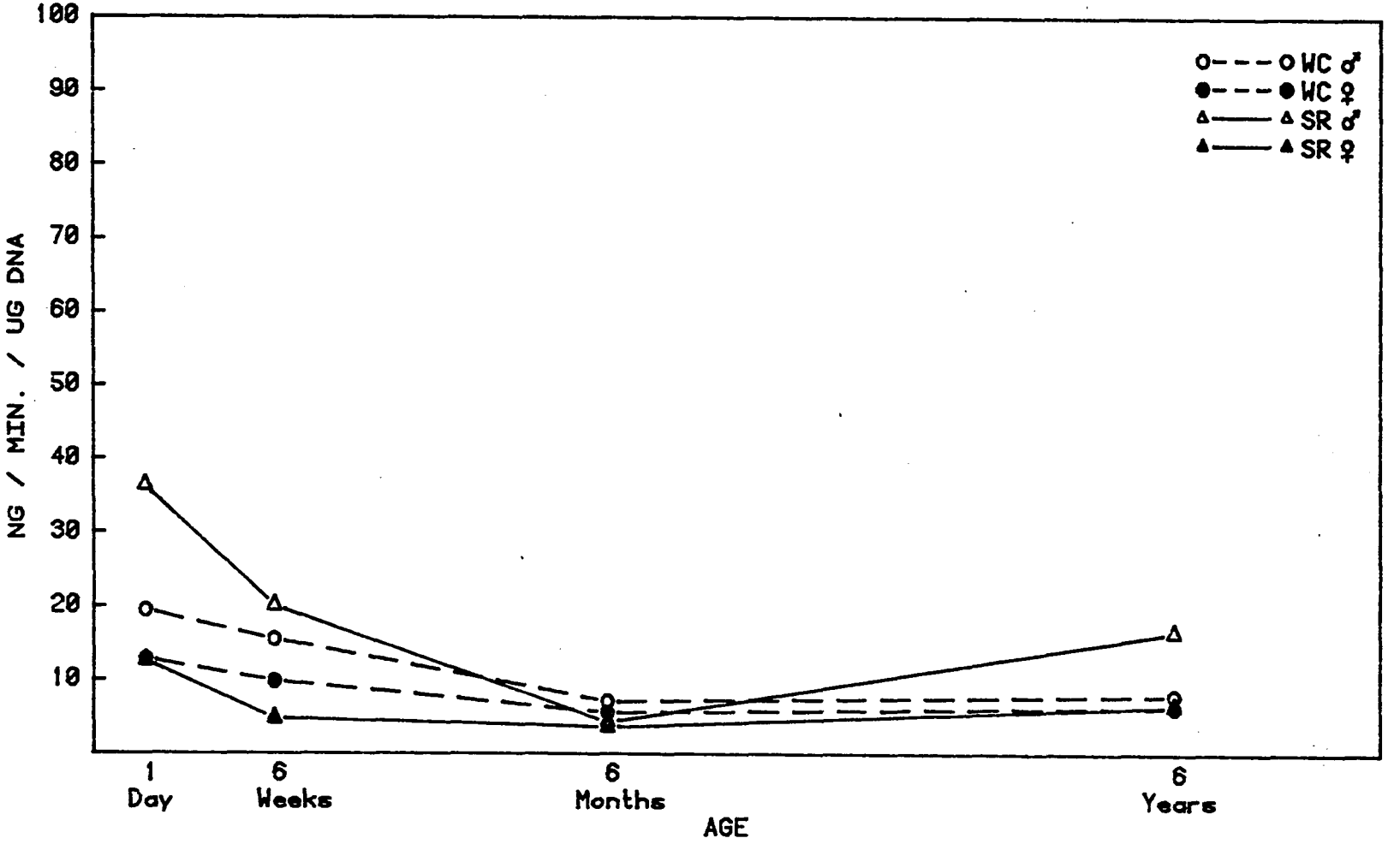


Figure 6e. Acid Phosphatase Activity in the Microsomal Fraction from the Upper Thoracic Aorta of White Carneau (WC) and Show Racer (SR) Pigeons.

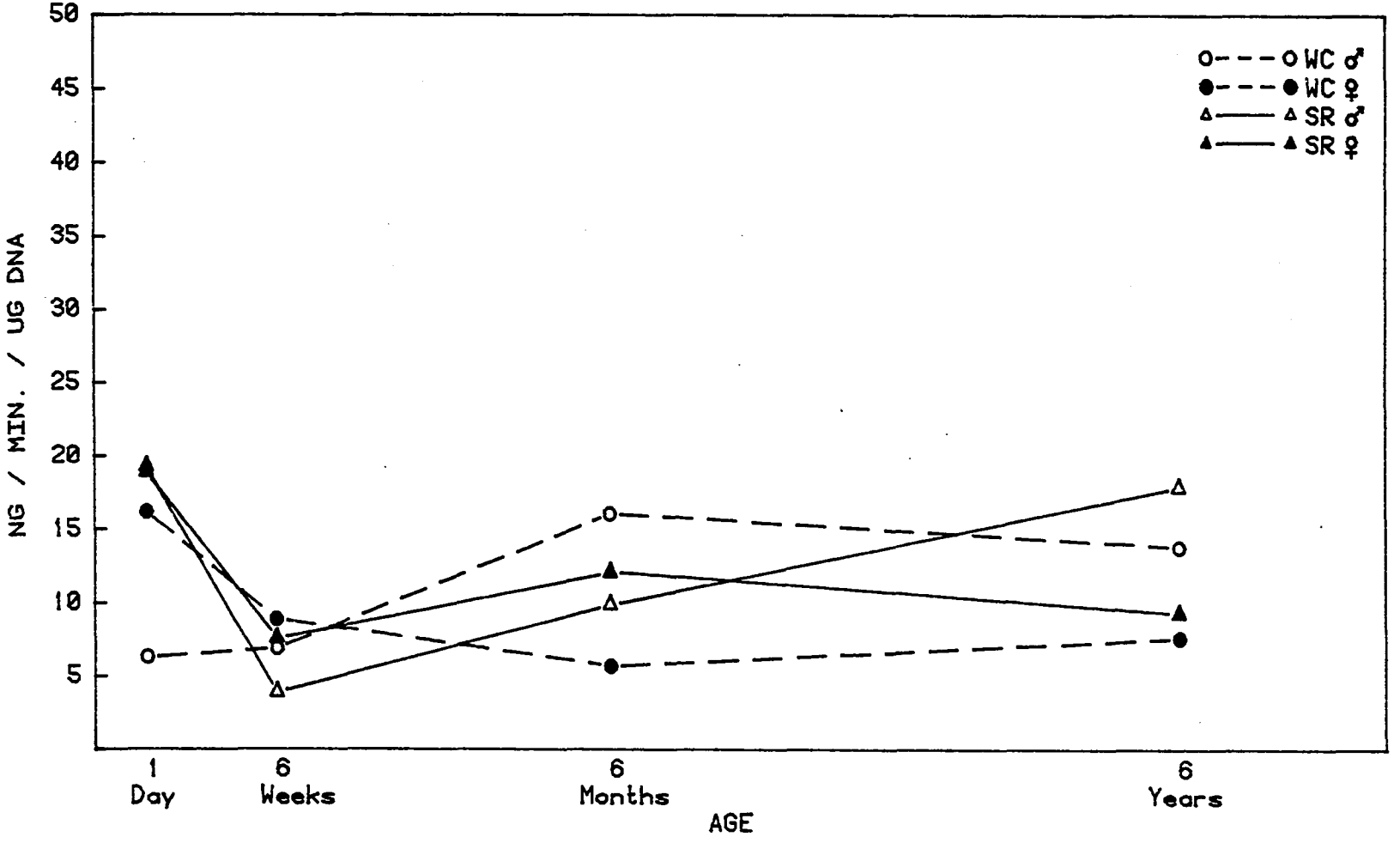


Figure 7a. Acid Phosphatase Activity in the Homogenates of the Celiac Bifurcation from White Carneau (WC) and Show Racer (SR) Pigeons.

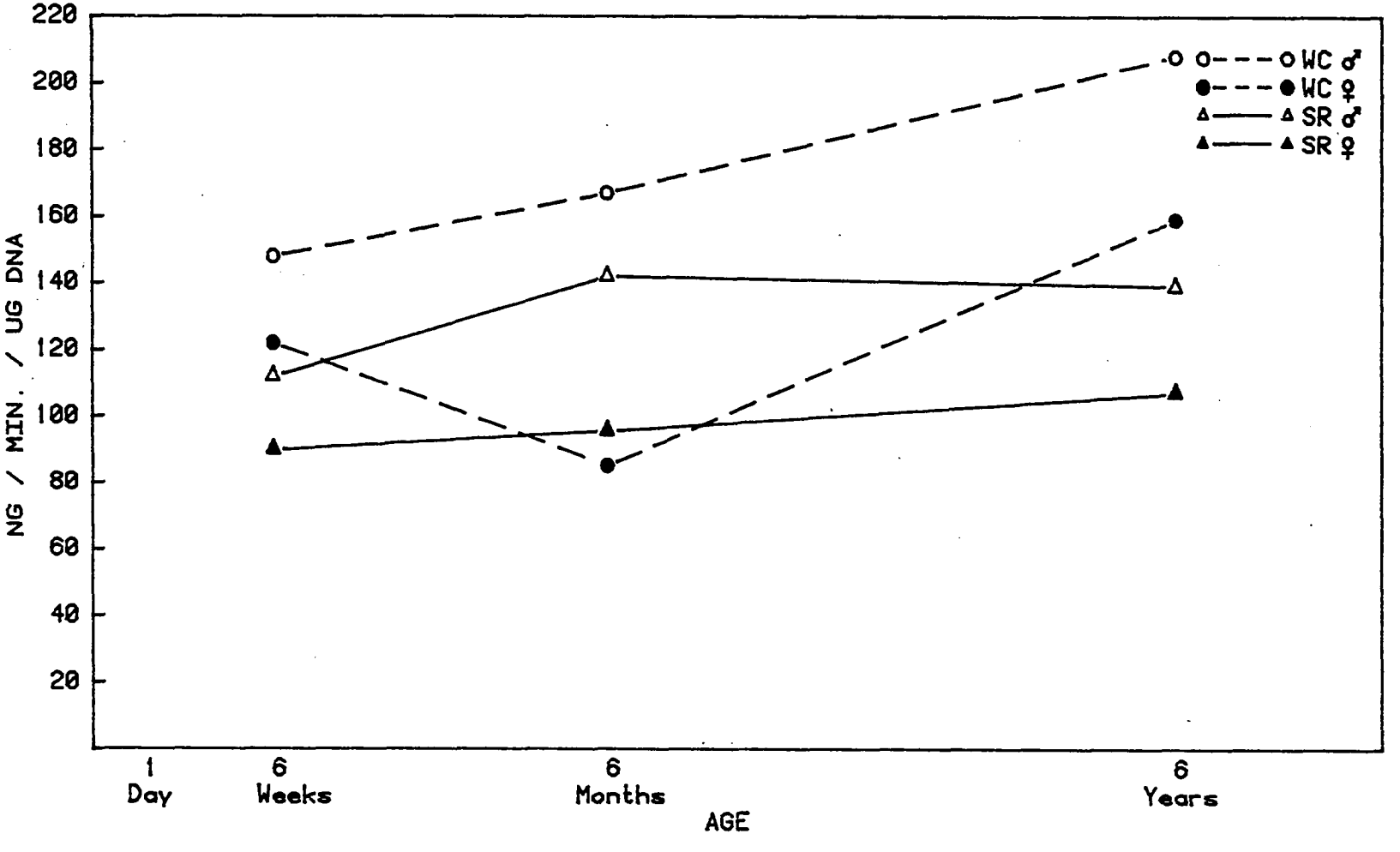


Figure 7b. Acid Phosphatase Activity in the Lysosomal Fraction from the Celiac Bifurcation of Female Pigeons.

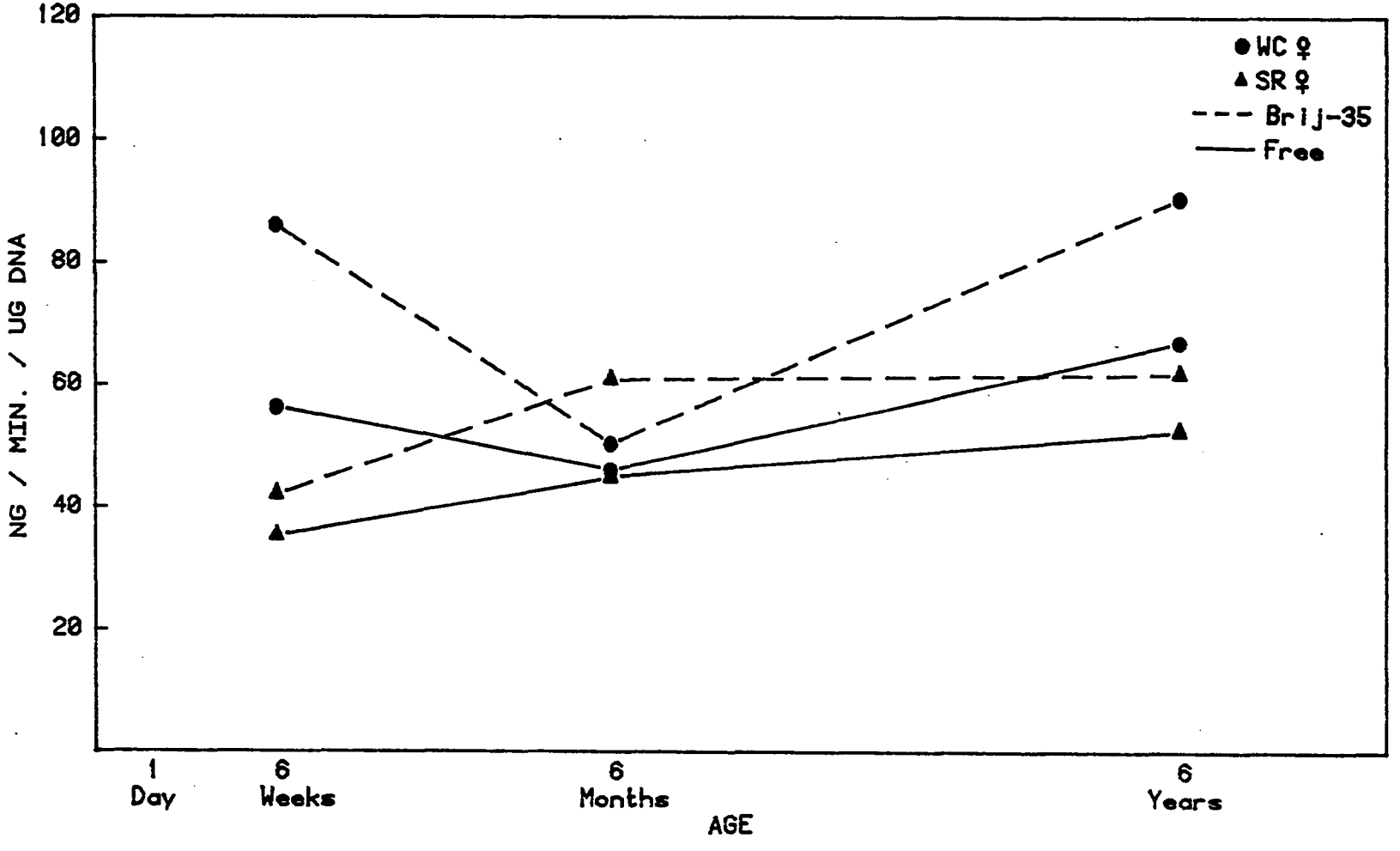


Figure 7c. Acid Phosphatase Activity in the Lysosomal Fraction from the Celiac Bifurcation of Male Pigeons.

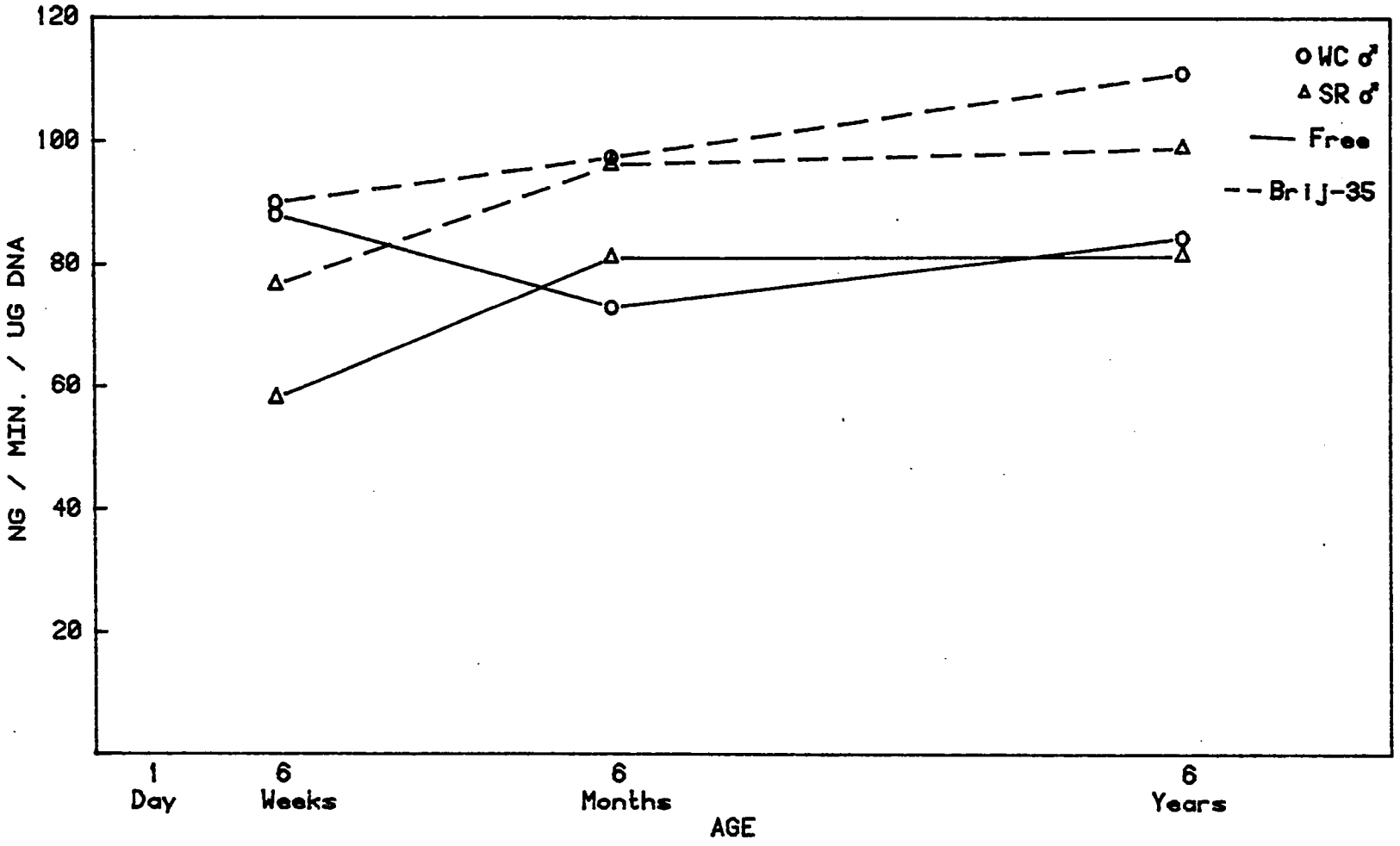


Figure 7d. Acid Phosphatase Activity in the Soluble Fraction from the Celiac Bifurcation of White Carneau (WC) and Show Racer (SR) Pigeons.

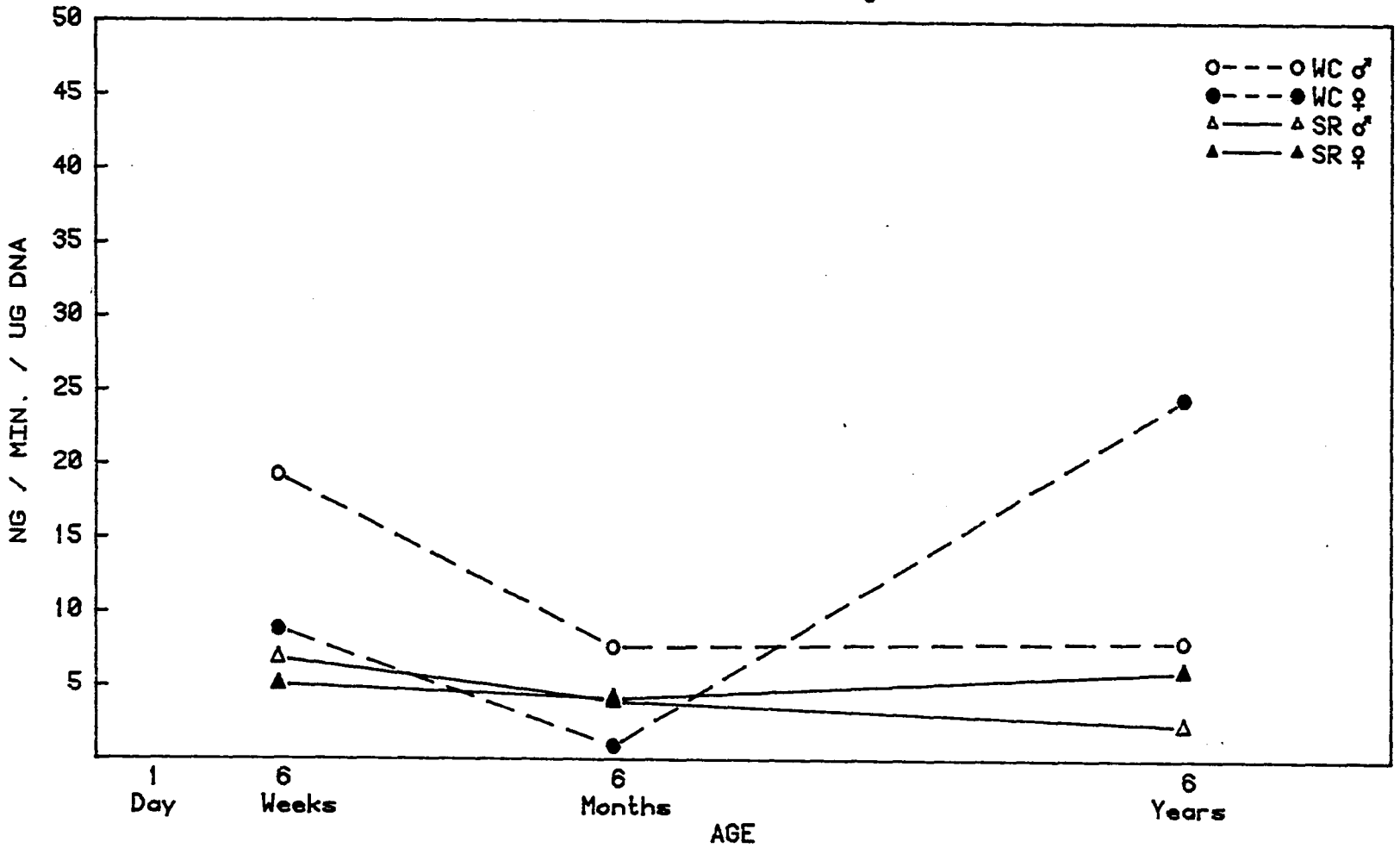


Figure 7e. Acid Phosphatase Activity in the Microsomal Fraction from the Celiac Bifurcation of White Carneau (WC) and Show Racer (SR) Pigeons.

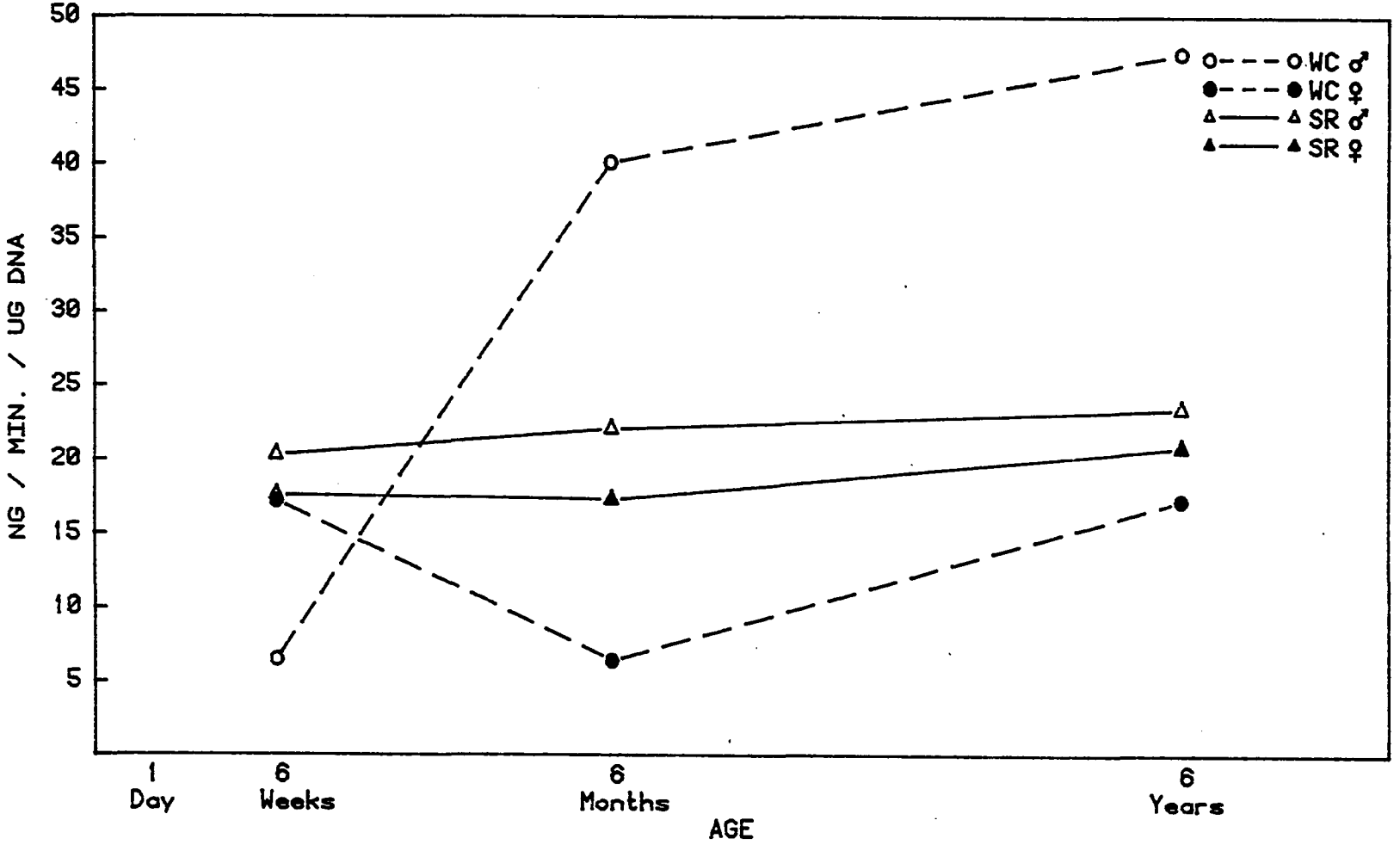
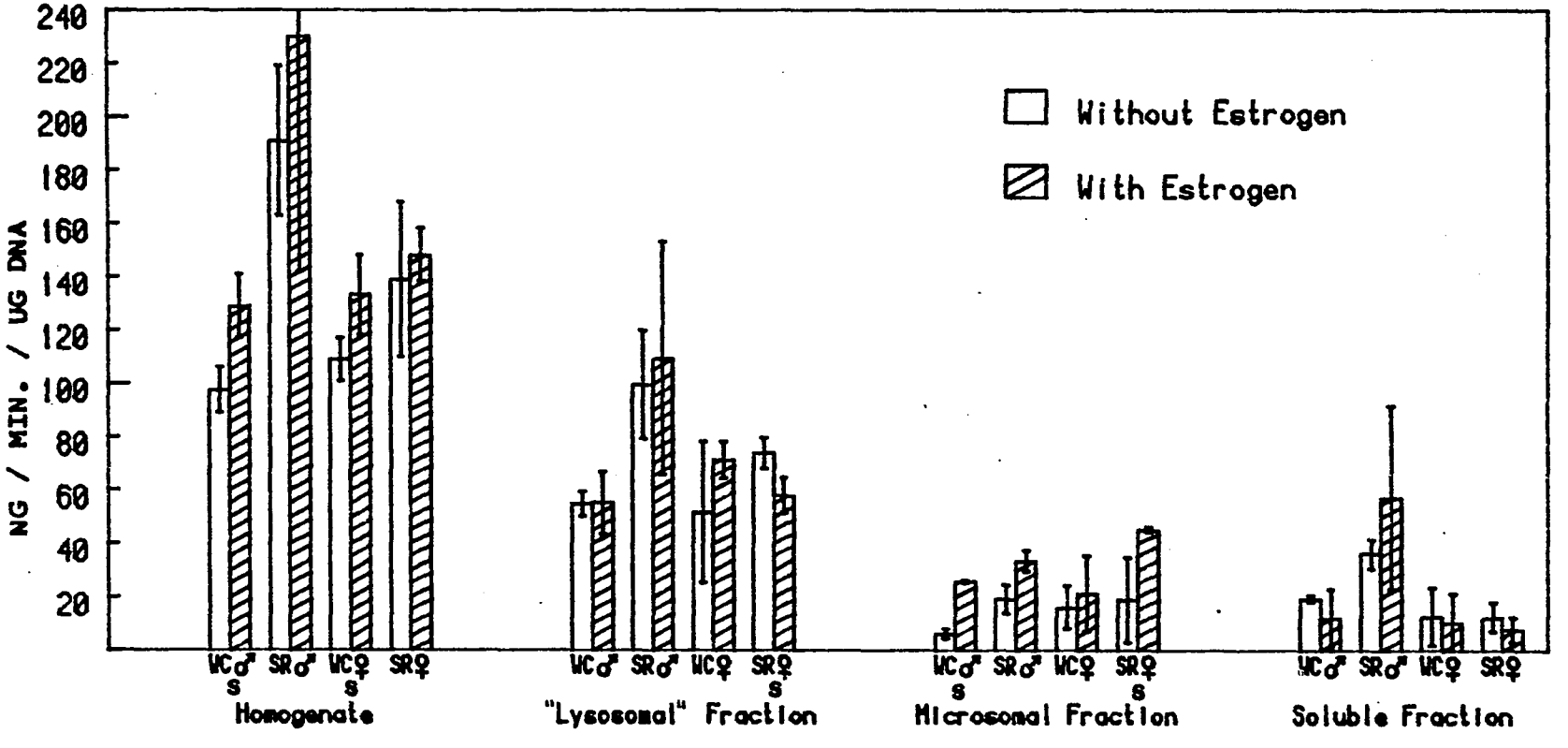


**Figure 8a**

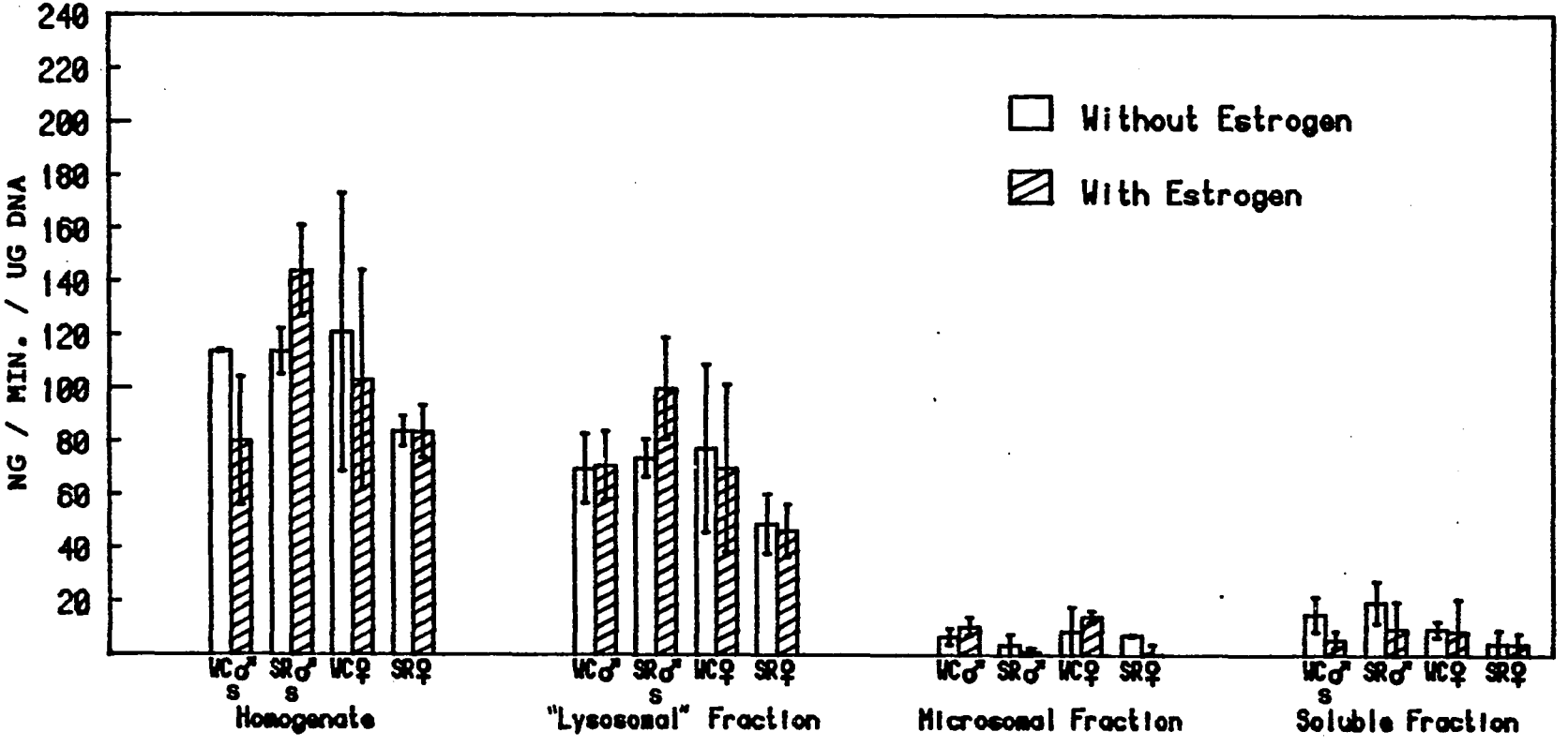
Acid Phosphatase Activity in the Thoracic Aorta of One Day Old White Carneau (WC) and Show Racer (SR) Pigeons with and without Estrogen Treatment.




s = significant difference ($P<0.05$) between treatments; statistical analysis was done by the Student's Test for paired samples.
Error bars represent ± 1 SD. WC=White Carneau; SR=Show Racer

Figure 8b

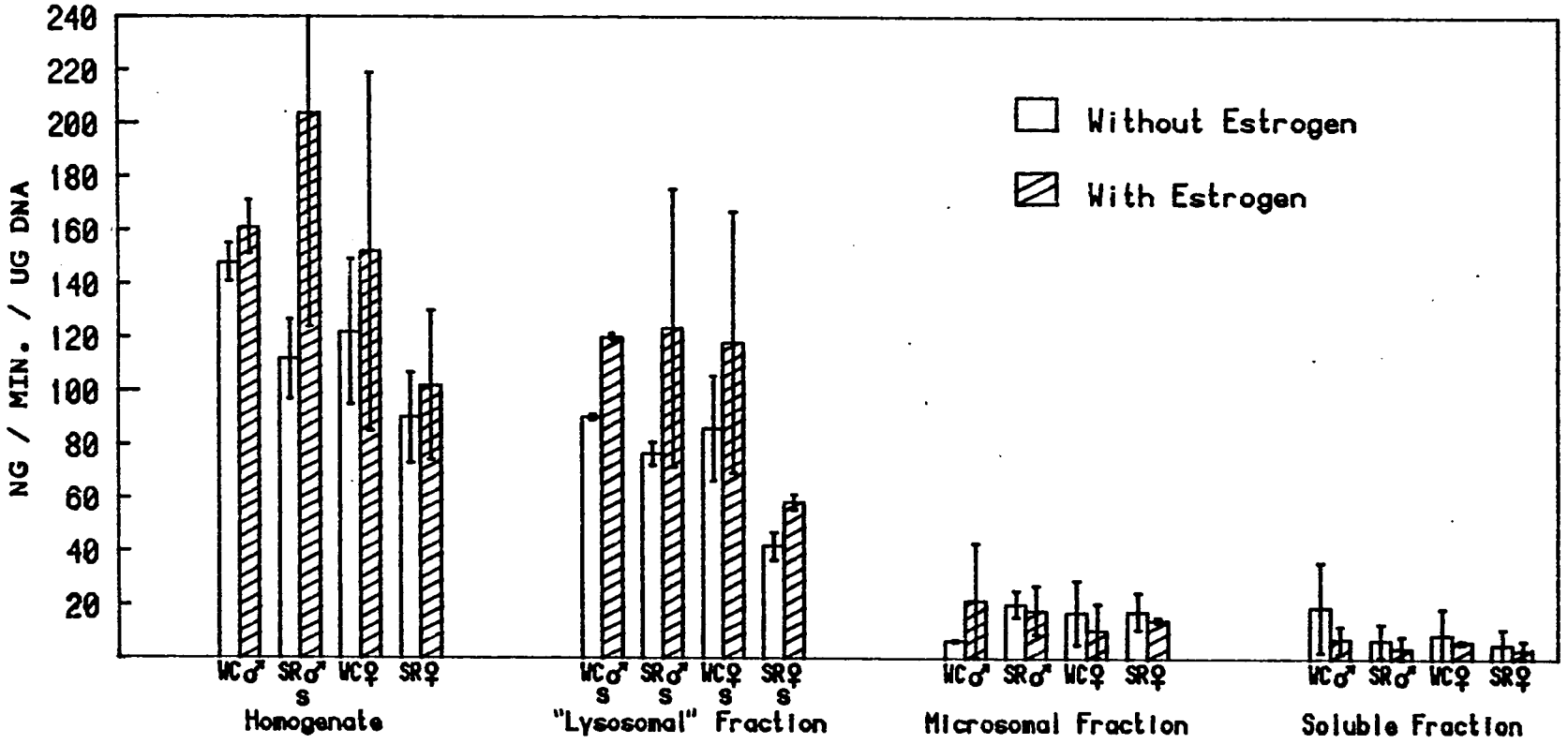
Acid Phosphatase Activity in the Thoracic Aorta of Six Week Old White Carneau (WC) and Show Racer (SR) Pigeons with and without Estrogen Treatment.




s = significant difference ($P<0.05$) between treatments; statistical analysis was done by the Student's Test for paired samples.
Error bars represent ± 1SD. WC=White Carneau; SR=Show Racer

**Figure 9a**

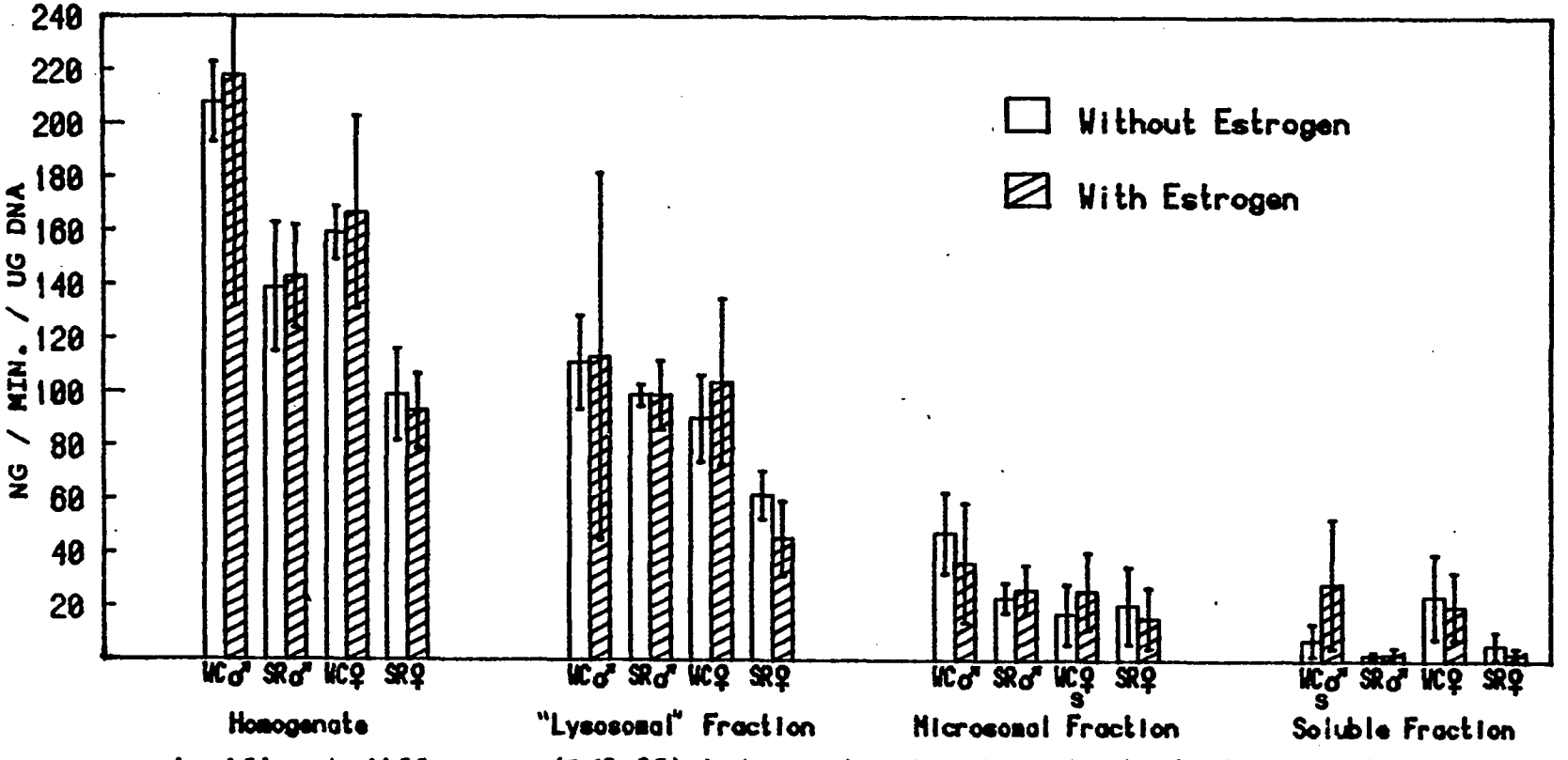
Acid Phosphatase Activity in the Celiac Bifurcation of Six Week Old White Carneau (WC) and Show Racer (SR) Pigeons with and without Estrogen Treatment.




s = significant difference ($P<0.05$) between treatments; statistical analysis was done by the Student's Test for paired samples.
Error bars represent ± 1 SD. WC=White Carneau; SR=Show Racer

**Figure 9b**

Acid Phosphatase Activity in the Celiac Bifurcation of Six Year Old White Carneau (WC) and Show Racer (SR) Pigeons with and without Estrogen Treatment.




s = significant difference ($P<0.05$) between treatments; statistical analysis was done by the Student's Test for paired samples.
Error bars represent ± 1 SD. WC=White Carneau; SR=Show Racer

## V. DISCUSSION

Acid phosphatase was formerly considered the most reliable lysosomal marker enzyme. Recently APase activity has been reported in other subcellular fractions (microsomal, nuclear, and soluble) (Bodansky, 1972; Nadler and Egan. 1970). Despite this fact it is still the most widely used enzyme as a lysosomal marker, and for this reason APase activity was measured along with NAHase (now considered the most specific lysosomal marker enzyme) activity in the present study.

It has been well established in various tissues from numerous animals that NAHase exists as two major isozymes, A and B. Isozyme A has been found in the soluble and lysosomal fractions of all homogenates before and after complete lysosomal disruption (Robinson and Sterling, 1968). Isozyme B, on the other hand, has been found only in the lysosomal pellet even after complete rupture of these organelles. This suggests that isozyme B is membrane bound whereas isozyme A is not membrane bound or is at least easily dissociated from the membrane. Recently Burnside and Schneider (1980) showed that NAHase in a purified lysosomal fraction is membrane found and can be displaced by mannose-6-phosphate. However, they did not do any determination of isozymes present in the membrane bound fraction. NAHase A is also much more sensitive than B towards heat inactivation (complete inactivation at $50^{\circ}C$ in 3 hrs.), and this is often used as partial criteria when identifing the isozymes (O'Brien *et al.*, 1970; Geiger and Arnon, 1976; Hayase *et al.*, 1973; Srivastava *et al.*,

1976). Freezing-thawing, acid, P-hydroxymercuribenzoate, and merthiolate have also been shown to inactive form A (Saifer and Rosenthal, 1973; Gieger and Arnon, 1976). Geiger and Arnon (1976) found that form B consisted of two non-covalently bound subunits of the same type each consisting of two "B" polypeptide chains linked by disulfide bridges. The A form consisted of two non-covalently bound subunits of two types, one with two "$\alpha$" polypeptide chains linked by disulfide bridges and one with two "B" chains identical to the B form subunit. The $\alpha_2$ and $B_2$ subunits are thought to be dissociated more readily than the $B_2$ and $B_2$ subunits. Human aortic tissue has been found to consist of 51% A and 49% B (Hayase et al., 1973). In the present study prolonged incubation at 41°C (60 min.), Triton x-100, digitonin, freezing-thawing, sonication, and Brij-35 were found to cause partial inactivation or inhibition of the NAHase activity in the soluble fraction. Almost half of the total aortic NAHase activity was found in the soluble fraction in both breeds. Since a large portion of the lysosomes were ruptured, and the soluble activity had an acid pH optimum, the soluble activity is most likely of lysosomal origin, may represent isozyme A, and was increased in severe atherosclerotic lesions (6 year old WC celiac bifurcation). Consistent with this idea is the finding that the A form of NAHase is increased in chronic liver disease as a response to mesenchyme reaction (i.e., acceleration of metabolism with increases in connective tissue, cholesterol, and/or fatty acid synthesis, cell proliferation, etc.) (Pott et al., 1978).

NAHase is known to be involved in the degradation of glycos-

aminoglycans (GAG) (by cleaving nonreducing terminal N-acetyl-glucosamine or N-acetylgalatosamine (Buddecke and Werries, 1965) Conehic and Levy, 1959) and has been found to be the rate-limiting factor in the degradation of hyaluronic acid (Hayashi, 1977; Weissman et al., 1975). Although NAHase activity levels have been shown to be dependent on sex (Hosoi et al., 1979) no sex differences in activity were seen at any age in the present study. However, NAHase activity did decrease significantly with age. This decrease in activity with age agrees with studies of NAHase activity in the rat aorta and decreased GAG turnover with age (Hermelin and Picard, 1978; Picard et al., 1974). However, NAHase activity in one day old thoracic aortas was much higher in the SR than in the WC, but dropped drastically in the SR by six weeks. The NAHase activity in the WC did not decrease to the same extent until 6 months. Since Curwin and Smith (1977b) have shown that amounts and profiles of GAGs in the thoracic segments of WC and SR pigeons do not change appreciably with age, synthesis of these macromolecules probably follows similar patterns of decrease with age as seen for NAHase activity. This suggests that synthesis of GAGs is higher in the WC at 6 weeks than in the SR, but since degradation is also higher (both tissue sites) actual amounts of GAGs present at 6 weeks in the WC and SR are not significantly different (both tissue sites). Supporting this idea is the recent report that WC smooth muscle cells in culture synthesize more GAGs than SR smooth muscle cells, but the relative proportions of the GAGs present are similar in the two breeds (Wight, 1980).

By 6 months, NAHase activity in the WC (both tissue sites) was similar to activity in the SR. However, total GAGs in the WC celiac bifurcation (6 months) were significantly higher than in the SR, suggesting that GAG synthesis remains high while degradative enzymes decrease. Stimulation of aortic smooth muscle cells to continue a high rate of GAG production may be caused by injury or alterations in extracellular matrix mediated by hemodynamic factors, infiltration of plasma constituents, or excessive release of lysosomal enzymes.

to Although much work has been done on NAHase isozymes, no one to date has looked at the specificity of these isozymes for different GAGs. Avila and Convit (1976) and Avila (1978) have shown that lysosomal enzymes interact with GAGs and that this interaction depends on pH, and degree and type of sulphation. However, they did not look at the specificity of separate isozymes. If, as indicated earlier, the relative distribution of NAHase activity reflects the relative isozyme pattern, then changes in the isozyme pattern (activity distribution) may alter individual GAG degradation or turnover. Consistent with this idea is the finding that although absolute amounts of NAHase activity in the thoracic segment differ at one day and 6 weeks between the two breeds, the distribution of activity is the same, and, as shown by Curwen and Smith (1977b) the GAG profiles are the same in the two breeds. However, in the celiac segment of the WC the NAHase soluble activity (isozyme A) increased from 6 months to 6 years. During this same time period total GAGs decrease but chondroitin-4-sulfate (Ch-4-$SO_6$)

decreased much more rapidly than other GAGs (Curwen and Smith, 1977b). This suggests that the soluble NAHase activity (form A) preferentially aids in the degradation of Ch-4-$SO_4$ relative to other GAGs. However, changes in synthesis of individual GAGs could also account for the observed differences.

APase activity in the SR (upper thoracic) decreased sharply with age (1 day to 6 weeks) as did NAHase activity. This suggests that there is a general decrease in the SR cellular catabolism. However, the WC (upper thoracic) showed no such decrease in APase activity with age. In fact, the WC male APase activity increased from 1 day to 6 months. A higher metabolic turnover rate is indicated in the WC than in SR as seen by high APase and NAHase activities. This high level could also be due to a response by the cell to replace acid hydrolases lost from the cell through excessive extracellular release (as discussed later).

Unlike NAHase, APase activity varied with sex. In the WC, sex differences appear to be amplified in the celiac segment when compared to the thoracic segment. This indicates either a greater inflex of circulating hormones (increased permeability of tissue or hemodynamic factors), or a greater response by cells in the celiac area to hormones. The SR did not show this difference between tissue sites. Furthermore, APase activities in the SR male and female showed the same general trend with age (only absolute values were different), but in the WC, APase activity variation with age was quite different between male and female. Both WC male and female had higher APase activity at 6 weeks in the celiac

than the respective SR pigeons. However, at this point activity in the WC male continued to increase to 6 years, whereas activity in the WC female dropped by 6 months and then increased by 6 years. Testosterone (major pigeon androgen, Sturkie, 1976) is known to cause increases in APase activity (Milone et al., 1978) as seen here in the WC and SR males upon reaching maturity (4-6 months). However, the increase in APase activity from 6 weeks to 6 months is significant only in the WC male suggesting that testosterone levels are higher in the WC or that the tissue response to testosterone is greater in the WC. This is supported by the fact that the SR males are less aggressive (Brannigan, 1973) and testosterone is believed to play a role in male behavior (Sturkie, 1976). Corticosterone (the major avian glucocorticoid) has been shown to decrease APase activity, so that an excess of corticosterone (produced by excessive stimulation of the adrenal caused by the stress of high reproductive activity (Brannigan, 1973), may be responsible for the large drop in APase activity from 6 weeks to 6 months in the WC female. This drop is not seen in the WC male, which has also been shown to have adrenal hyperplasia; however, the opposing effect of testosterone on APase activity may have eliminated the effect of corticosterone.

Subcellular fractionation showed that enzyme localization at 6 years was depentent on sex. Both sexes in the WC showed increased APase activity in the "lysosomal" fraction; however, activity in the male also increased in the microsomal fraction but not in the soluble fraction. The activity in the WC female increased in the sol-

uble fraction but not in the microsomal fraction. Treatment of celiac tissue of WC males with estrogen caused a shift of activity from the microsomal to the soluble fraction. This shift could be due to lysis of the microsomes and/or release of membrane bound activity, or an increase in fusion of enzyme containing particules with lipid-laden particles. Sergeer et al. (1978) found that estrogen increased the release of APase and inhibited membrane binding of enzymes.

Since lysosomes are the major site of catabolic activity within the cell, increased lysosomal acid hydrolase activity from 6 months to 6 years in the WC celiac segment may be a response by aortic smooth muscle cells to increases in substrates, i.e. connective tissue components, lipids, cell debris, and plasma constituents. The accumulation of such substances suggests a defect in catabolism by overloading (synthesis and/or endocytosis) of the cell's vacuole system, by inhibition or alterations of degradative enzymes, and/or by alterations in the lysosomal, endocytotic vacuole, or autophagic vacuole membranes. Increases in acid hydrolases were not as dramatic as seen in cholesterol fed animals (Peters et al., 1972; Peter and de Duve, 1974) nor was this increase predominately seen in the "lysosomal" fraction. This suggests that the spontaneous lesion is biochemically as well as morphologically (Curwen and Wight, in preparation) different from the cholesterol- fed lesion.

High yields of intact aortic lysosomes are precluded because of the large amount of connective tissue present in the vessel wall. Peters et al., (1972) and others have used collagenase,

hyaluronidase, and elastase to digest the connective tissue matrix of minced aortic tissue in order to obtain intact cells for subsequent fractionation and, therefore, obtained a higher yield of intact lysosomes. However, when this was attempted with pigeon aortas, the total NAHase activity decreased appreciably, while APase total activity increased. Presumably NAHase was denatured or degraded during the (1½ to 2 hrs) incubation of the tissue. Furthermore, the enzyme mixture used to digest the tissue was found to contain acid phosphatase activity which was apparently partially retained by the aortic tissue even after several washings. In the present study, fine mincing and gentle homogenization of the tissue gave the highest yield of intact lysosomes without alterations in enzyme activity. Even so, the yield of intact lysosomes obtained was low.

Latency of lysosomes can be measured in two ways: the % acid hydrolase activity sedimented by ultracentrifugation; or the measurement of activity before (free activity) and after (total activity) disruption of the lysosomal membrane. It is well known that some of the "free" acid hydrolase activity measured in a tissue homogenate will sediment with intact lysosomes (Davies, 1975); therefore, measurement of the % activity sedimented would not give an accurate measurement of lysosomal latency or fragility. On the other hand, methods used to disrupt lysosomes (detergents, freezing, and thawing) can alter acid hydrolase activity through direct inhibition or denaturation of the enzymes. Since 40-50% of the NAHase activity was found in the soluble fraction in the pre-

sent study, then at least 40-50% of the lysosomes containing NAHase were ruptured during homogenization. However, only 20-40% of the activity sedimented in the "lysosomal" fraction was latent. This low latency in the "lysosomal" fraction could be the result of one or two processes: the activity is bound to large lysosomal membrane fragments formed during homogenization, or the majority of the lysosomes were intact, but were ruptured during ultracentrifugation. Nevertheless, after taking into account the activity in the soluble and microsomal fractions, latent NAHase activity in the homogenate was similar to that seen in the "lysosomal" fraction. Therefore, the free activity in the "lysosomal" fraction is probably due to membrane bound enzymes of lysosomes ruptured during tissue homogenization. The latent activity seen was probably due to primary or small secondary lysosomes better able to withstand homogenization than larger secondary lysosomes.

The latent APase activity is quite variable and hard to interpret. Since APase activity is sex dependent the differences in latent activity between sexes may simply be a reflection of biochemical heterogenity of lysosomes (Davies, 1975). However, APase is no longer considered a reliable lysosomal marker enzyme because it can be detected in other subcellular fractions, particularly in the microsomes. Complete separation of lysosomes from microsomes is difficult, and was not attempted in this study. (Nor was the degree of microsomal contamination of the lysosomal fraction assessed.)

Van Dijk *et al.*, (1976) determined that 17% of the protein present in a "lysosomal" fraction (sedimented at 25,000 g in 0.25

M sucrose) was actually due to the presence of microsomes. Although samples were spun at 20,000 g to obtain a "lysosomal" fraction in the present study, the presence of microsomes in the "lysosomal" fraction is likely. Furthermore, WC aortic smooth muscle cells in tissue culture (Wight et al., 1977) and in vivo (Cooke and Smith, 1968) have a strikingly higher amount of dilated ER than SR aortic smooth muscle cells. Upon homogenization a larger proportion of the dilated ER from the WC may have sedimented with the lysosomal fraction. Therefore, part of the APase activity seen in the "lysosomal" fraction may be actually due to activity present in the endoplasmic reticulum within the intact cell. Supporting this idea is the fact that latent APase activity seen in the "lysosomal" fraction from celiac segments of WC can be correlated with the activity seen in the microsomal fraction such that when structural latency was seen in the microsomal fraction and the activity was high, latency was also seen in the "lysosomal" fraction(eg. in 6 month old WC male celiac segment). However, when activity in the microsomal fraction was low, latency in the "lysosomal" fraction was low or not significant (eg. in 6 week old WC male celiac). When estrogen treatment appeared to stabilize the lysosomes (increase in latency as seen in 6 week old WC male celiac), the microsomal activity also increased. Therefore, differences in "lysosomal" APase activity and latency may be partially due to differences in microsomal activity and can not be used as representing only the lysosomal activity. NAHase activity on the other hand, has not been detected cytochemically in the endoplasmic reticulum or biochemically in microsomal preparations. Therefore, latent

NAHase activity is probably representative of lysosomal stability. The small amount of NAHase activity found in the microsomal fraction in this study was probably due to small primary lysosomes, or more likely, since no significant structural latency was seen, due to NAHase bound to small lysosomal membrane fragments.

Despite the variation in the latent NAHase activity present in the homogenate and "lysosomal" fractions, significant differences in latent activity between breeds were seen. In the 6 week old WC, no significant latent NAHase activity is seen at either tissue site, indicating a greater lysosomal fragility in the WC than in the SR. The percentage and actual amount of latent activity in thoracic segments were not significantly different between breeds. However, estrogen treatment did decrease latency in the WC and increase it in the SR, so that the percentage of latent activity was significantly higher in the SR than in the WC. In the celiac segment of the WC lack of structural latency was more evident and was significantly lower than in the SR. Estrogen treatment appeared to stabilize the lysosomes from the WC celiac, so that the difference between the SR and WC latency is not statistically significant.

The effect of estrogen treatment on lysosomal latency varied between tissue sites in the WC and between breeds. The different responses may be due to the interaction of the estrogen molecule with lysosomal membrane components, and, as is seen for cholesterol, the actual individual membrane components (phospholipids in particular) would determine the overall effect (stabilization or labilization) of estrogen on the membrane. At 6 months estrogen treatment caused a

complete disappearance of latency in SR celiac segment. This may reflect changes in the lysosomal membrane components at 6 months in the SR, or an accumulative effect of estrogen (endogenous estrogen present prior to the removal of tissue and the added exogenous estrogen (*in vitro* treatment)).

The most widely accepted model of the mode of estrogen action is reviewed by Schulster *et al.* (1976). In the model, estradiol diffuses into the cell and combines non-covalently and specifically with a receptor protein present in the cytoplasm. The estrogen-receptor complex then moves into the nucleus, where it is thought to affect specific transcription processes at the level of the gene. However, Szego (1976) has described a model where estrogen action is mediated by lysosomes. In this model estrogens are taken up by the cell through endocytosis rather than by passive diffusion through the membrane. The endocytotic vesicles fuse with lysosomes which contain the estrogen receptor. The lysosomes containing estradiol then move into the nucleus where specific transcription processes are affected. The fact that tritiated 17B-estradiol was taken up by aortic tissue more rapidly in buffer containing glucose than in buffer without glucose or with ethanol (unpublished observations) suggest that an energy requiring process does at least aid in the uptake of estrogens and may be selective since the uptake of 17B-estradiol was ten times that of estriol. However, estrogen treatment did not alter lysosomal latency in a consistent predictable pattern as reported by Szego (1971, 1976) and Szego *et al.*, (1972). This discrepancy may be due to differences in the actual amount of estrogen taken up by the tissue or in the responses of reproductive versus non-reproductive organs.

It has been shown that increased fragility (or destabilization) of membranes enhances membrane fusion (Ahleong et al., 1975; Poste and Allison 1973). Activation (or destabilization) of lysosomes in tissue has been shown to cause breakdown of extracellular matrix (Reynolds, 1969) by excessive release of acid hydrolases. Such a phenomenon in pigeon aorta would cause localized alterations in the connective tissue matrix surrounding the cells, changing tissue permeability and binding characteristics. Excessive lysosomal fragility may also result in leakage of enzymes into the cytosol or cause excessive autophagy leading to cell degeneration or death. Hajjar et al., (1980) recently reported significantly higher amounts of nonesterified fatty acids (NEFA) and extracellular debris-like material in the celiac bifurcation of the 6 week old WC than in the celiac bifurcation of the SR. Cooke and Smith (1968) have also shown a loss of extracellular matrix, in particularly collagen and elastin. NEFA have been shown to decrease lysosomal membrane stability (Raz and Goldman, 1976); therefore, increases in NEFA seen in the 6 week old WC celiac bifurcation may cause destabilization of lysosomes which in turn may cause extracellular matrix and cell degeneration. Since low membrane stability is also seen in the thoracic segment in 6 week old pigeons as well, a genetic origin of membrane fragility cannot be overlooked. It is presently thought that membrane integrity and function depend on the lipid composition of the membrane (Cullis and DeKruuff, 1979). Furthermore, different phospholipids prefer different configurations. Phosphatidylethanolamines prefer a hexagonal arrangement (considered to be an intermediate configuation during membrane fusion), but phosphatidylcholine and sphingomyelin prefer the more stable bilayer

configuration. When either phosphatidylcholines or sphingomyelins (at more than 30 mole %) are added to phosphatidylethanolamines (in a non-bilayer configuration), the bilayer phase is induced. In mixed lipid systems (biological membranes) cholesterol can stabilize the bilayer structure or disrupt it depending on the degree of saturation of the fatty acids present. The addition of $Ca^{2+}$ can also trigger the formation of the hexagonal configuration. Differences in the phospholipids, cholesterol, and fatty acids components of lysosomal or other cellular membranes may be responsible for differences in lysosomal membrane stability between the SR and the WC. A greater influx of plasma components (cholesterol, phospholipids, $Ca^{2+}$, hormones), which would be endocytosed by the cell and subsequently fuse with lysosomes, in the WC celiac bifurcation than in the thoracic segment due to hemodynamic factors may increase the membrane fragility even more, inducing extracellular and intracellular release of lysosomal enzymes, and causing the damage described earlier.

Cramer and Smith (1976) suggested that the slower rate of utilization of yolk phospholipids by WC embryos may reflect a difference in the composition of the phospholipids. Furthermore, Hajjar et al., (1980) also reported increased levels of phospholipids in one day old WC embryos. Hajjar and Smith (1980, 1978) reported that in 6 week old WC celiac segment there is a lack of ATP regulation of NADH transhydrogenation and suggest that in 6 month old WC celiac segments low P/O ratios represent uncoupled respiratory - chain phosphorylation. It has been reported that excessive release of lysosomal enzymes into the cytosol may be involved in the disruption of mitochrondrial function by inhibiting $Ca^{2+}$ uptake by the mitochondria or by uncoupling oxidative

phosphorylation (Lefer, 1976). Disruption or leakage of lysosomes would release phospholipases which could damage the mitochondrial and other membranes producing nonesterified fatty acids and lysophospholipids (surface active agents which could cause further damage) (Weglicki et al., 1974). Uncoupled oxidative phosphoralation may be responsible for a decrease or depletion of ATP leading to high intracellular $Ca^{2+}$ causing "blebbing off" of the plasma membrane (seen by Hajjar et al, (1980), in 6 week and 6 month old WC celiac segments) as was seen in the erythrocyte on ATP depletion (Sheetz and Singer, 1977; Lutz et al., 1977). Hajjar and Smith (1980) suggested that lack of ATP regulation of NADH transhydrogenation and other impaired mitochondrial functions may enhance lipid biosynthesis and exacerbate the development of atherosclerosis in WC pigeons.

Both tissue sites show an increase in saturated to unsaturated FA ratios from 6 weeks to 6 months. This may be in part a response by the cell to increase membrane stability (Papahadjopoulos, 1974) rather than completely due to lower oxygen levels found at 12 weeks in the celiac of WC (Farber, 1978).

WC celiac smooth muscle cells may respond to this intra- and extra-cellular injury (caused by excessive release of acid hydrolases) by elaborating different types or proportions of GAGs as seen by 6 months of age (Curwen and Smith, 1977b). Alterations in GAG profiles coupled with hemodynamic factors could cause increased sieving and retention of plasma constituents (esp. Lipoproteins within the vessel wall as well as influence metabolic pathways within the smooth muscle cell itself (Hay and Meier, 1974). GAGs (esp. Ch-6-$SO_4$) have been shown to interact with lysosomal acid hydrolases (Avila and Convit,

1975, 1976) and this interaction varies with degree and type of sulfation (Avila, 1978). GAGs have also been shown to cause abnormal isozyme distribution of acid hydrolases (Kint et al., 1973). Curwen and Smith (1977) have suggested that a variant type of heparitin sulfate (HS') present in the WC celiac segments may have a higher sulfate content than HS present in the WC thoracic or in the SR accounting for its higher electrophoretic mobility. Therefore, interaction of this variant HS' with lysosomal hydrolases may be different from interactions with normal pigeon HS and contribute to altered catabolic function in the WC celiac segment. Increased deposition of plasma constituents causing increased smooth muscle cell endocytosis leading to overloading or alteration of the cells degradative pathway combined with hydrolase alterations may be major contributing factors to lesion progression in WC pigeons.

Even though lysosomal membrane stability increases by 6 months (possibly due to increases in saturated fatty acids or increased hormonal influences (adrenal corticosteroids stabilize lysosomal membranes)), connective tissue matrix alterations and mitochondrial damage already exist and can cause lesion progression.

Further studies on lysosomal membrane stability are needed to confirm the lysosomal fragility seen in the WC celiac foci at 6 weeks. experiments of this type would best be carried out on aortic cells in culture since a high yield of intact lysosomes could probably be obtained because of the lack of extensive connective tissue matrix. Analysis of individual phospholipids present would also aid in the determination of the role of membrane structure and fragility in atherogenesis.

The protein yields of the "lysosomal" fractions include protein from lysosmes, mitochondria, and possibly microsomes, so that differences between breeds, sexes, ages, and tissue sites are difficult to interpret. Even though the protein content of lysosomes is not thought to be constant (Davies, 1975), alterations in protein yield of a lysosomal fraction may reflect changes in the number of lysosomes as well as increased or decreased content of acid hydrolases (especially in the 6 week old WC male). The effects of estrogen treatment on the "lysosomal" protein yield were similar to the effects on APase activity. This suggests that in 6 week old pigeons (except SR female) estrogen treatment increased the number and/or enzyme content of lysosomes. Decrease in protein yield may reflect a decrease in protein synthesis which would decrease formation of lysosomes, so that the number of lysosomes would diminish as they became inactive. Increases in microsomal protein yield and APase activity is suggestive of newly synthesized acid hydrolases. An increase in transcription subsequent to estrogen administration (in vivo and in vitro) is one of the first responses of target tissues, however a general increase in protein synthesis is not seen for another 2-3 hours (Schulster et al., 1976; Gorski et al., 1975). Therefore the effects of estrogen treatment on protein yields (and enzyme activities) in the present study may not be due to general synthesis of RNA and protein, but rather to an early selective effect on RNA and protein synthesis. Szego (1972, 1976) and Szego et al. (1971, 1977) suggested that lysosomes containing the estrogen receptor, translocate the estrogen-receptor complex to the nucleus where the lysosomal acid hydrolases aid in intranuclear penetration. However, their emphasis was on lysosomal membrane labilization by steroids rather

than changes in actual enzyme activities. Therefore, rapid (within 30 min.) alterations in acid hydrolase activity, subcellular location (seen at 6 years in the WC celiac segment), and/or lysosomal membrane stability (not seen consistently here) may be a result of estrogen action on the cell, but may not play a role in the initiation of the cellular response to estrogen. Furthermore Sierralta et al. (1978) suggest that lysosomes are involved only in the degradation of the estrogen-receptor complex. However, these proposed roles of lysosomes in estrogen action were suggested for organs in the reproductive system, and even though estrogen receptors have been reported in the arterial wall (Stumpf and Sar, 1977), the effect and mode of estrogen action may be quite different than that which occurs in cells of the reproductive system, even smooth muscle cells.

The major effect of estrogen treatment on enzyme activities occured at 1 day and 6 weeks, prior to sexual maturity with the SR male being the most responsive. Generally activities increased; however, subcellular fractionation showed a different distribution of activity depending on sex, age, breed, and tissue site. These differences in response of the tissue to estrogen suggest that other factors (hormones present prior to removal of tissue, or tissue uptake or sensitivity) may alter the effect of estrogens on enzyme activities. This type of developmentally related "responsiveness" of lysosome enzyme activities to hormones has been seen in humans, rats, and mice (Oberkotter et al., 1980a; Oberkotter et al., 1980b; Swank, 1978). Swank (1978) reported that growth hormone potentiated responsiveness of B-glucuronidase to androgenic stimulation. Therefore, differences in levels of estrogens, testosterone (shown to augment estrogen stimulation

of phospholipid synthesis in rat aorta (Chobanian, 1968)), glucocorticoids (known to antagonize estrogen action (Szego, 1972) or other hormones are suggested and may be responsible for variations in tissue response. Sex differences in APase activity occur at one day in the SR, but not until later in the WC, further suggesting differences in circulating hormones between the male pigeons. APase activity is extremely high in the immature SR male and estrogen treatment increases it still further, suggesting that the SR male may have higher levels of estrogens at this age than the WC male. Unfortunately, preliminary attempts to measure estrogen levels in the birds used in this study were not successful.

At 6 weeks in the celiac segment of all birds, APase activity increased in the lysosomal fraction. This preferential increase in the lysosomal fraction rather than other fractions (as seen in advanced atherosclerotic lesions) suggests that estrogens may aid in lysosomal formation as well as increase synthesis of acid hydrolases. Since lysosomes are essential for degradation of cellular components, increased number of lysosomes would aid in the degradation of intracellular lipids which accumulate in atherosclerosis and may provide some "protection" against lesion development. Supporting this idea is the observation that estrogen administration suppresses the connective tissue accumulations seen in hypertensive rat aorta (Wolinsky et al., 1974). However, estrogens may alter connective tissue synthesis as well.

Estrogen may also elicit responses in aortic smooth muscle cells not seen here because of the short treatment time. Chobanian (1968) reported that estrogen treatment (in vivo and in vitro) increased

phospholipid synthesis in the arterial intima; however, individual phospholipids were not analyzed. Perhaps estrogen stimulation of individual phsopholipid synthesis may change the phospholipid composition of the lysosomal membrane, thereby altering membrane stability.

Studies on the effect of *in vivo* estrogen administration on atherosclerotic lesion development in the WC suggest that estrogens provide a "protective" effect only when administered to young birds and do not promote lesion regression in 6 year old birds (Souadjian *et al.*, 1968, and Hanash *et al.*, 1972). The greater response to estrogen in the tissue from immature birds seen in the present results may explain why only young birds show a "protective" effect of estrogens. On the other hand, once alterations in the connective tissue matrix and mitochondrial function (caused by excessive release of acid hydrolases due to fragile lysosomes at 6 weeks of age) occur, atherosclerotic lesions may progress despite administration of estrogen, alterations in membrane phospholipid composition, lysosomal membrane stabilization, and stimulation of lysosomal and microsomal enzymes.

## VI. CONCLUSIONS

Differences in lysosomal enzyme activity and membrane stability between aortas in atherosclerosis-susceptible WC and atherosclerosis-resistant SR pigeons have been shown to occur at early ages. However, their relationship to initiation or development of atherosclerotic lesions remains to be clarified by assay of other enzymes involved in lipid and connective tissue degradation, and by re-evaluation of associated factors which may affect lysosomal stability (ie. NEFA levels, membrane phospholipid composition).

Specific inferences are as follows:

1. Higher lysosomal enzyme activities and protein yields occur in both aortic sites of 6 week old WC indicating an increased capacity for catabolism. Since no appreciable accumulation of GAG or lipid is seen at this age, a higher turnover rate of intracellular and extracellular components is indicated.

2. At 6 weeks of age lysosomes (or a population of lysosomes in the WC aorta) are more fragile than in the SR. Excessive release of acid hydrolases due to fragile lysosomes correlates with connective tissue breakdown, GAG accumulation, and altered mitochondrial function seen in WC celiac segments. Altered lysosomal fragility may also deplete the cell's vacuole system of acid hydrolases, thereby decreasing catabolism of cellular components and endocytosed material (eg. lipid) to enhance lipid accumulation.

3. Although differences in lysosomal enzyme activities and fragility are seen between the two breeds of pigeons at 6 weeks in both

tissue sites, differences are greater between the celiac segments than between the thoracic segments. This indicates that local factors may accentuate metabolic alterations which lead to celiac lesion development in WC.

4. The greatest and most consistent effect of estrogen treatment occurred in 6 week old celiac segments where lysosomal APase activity and protein yield increase in both sexes of each breed. These increases may reflect an increase in the number of lysosomes and/or activity of acid hydrolases. Such an increase in the cellular catabolic capacity in tissue sites predisposed to atherosclerotic lesion development may explain the reported estrogen "protection" against lesion development.

5. The diversity of tissue response to estrogen treatment between breeds, sexes, and ages and the greater response of the celiac segment indicates that local factors accentuate metabolic alterations and responses.

6. APase activity in the arterial wall of SR and WC pigeons is higher in males and than in females of the same age, and sex differences occur as early as one day in the SR. However, the increase in APase activity seen in males upon reaching maturity is less in the SR than the WC so that differences between sexes are then similar in both breeds. This may indicate a different mechanism of disease progression in WC males and females since lesion severity is similar in the birds. NAHase activity is not sex related.

7. Increase of soluble NAHase activity at 6 years in the WC celiac segment suggests a disproportionate increase in isozyme A relative to isozyme B which may alter GAG degradation and explain previously reported changes in arterial wall GAG composition. Since the increase

in soluble activity does not occur until after 6 months of age, and then only in the celiac segment, it is most likely a consequence of lesion development and not an initiating factor. However, it may still contribute to lesion progression.

8. Since the greater lysosomal enzyme activities, protein yields and membrane fragility in the WC occur at both tissue sites a genetic mechanism is indicated. As mentioned earlier (number 3 above), higher lysosomal enzyme activities, protein yields, and membrane fragility in the WC celiac segment appear to be augmented by local factors, and may play a major role in spontaneous atherogenesis.

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# APPENDIX

Table 1.

Protein Yield of "Lysosomal" and Microsomal Fractions as a Function of Age and Sex in White Carneau (WC) and Show Racer (SR) Aortic Sites.

| | | "Lysosomal" Fraction | | | | Microsomal Fraction | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Thoracic | | Celiac | | Thoracic | | Celiac | |
| | | WC | SR | WC | SR | WC | SR | WC | SR |
| No treatment | | | | | | | | | |
| 1 day | M | 2.97 ± 0.41 | 2.94 ± 0.82 | | | 0.31 ± 0.17 | 0.55 ± 0.14 | | |
| | F | 2.31 ± 0.52 | 3.39 ± u.50 | | | 1.06 ± 0.50 | 1.67 ± 1.30 | | |
| 6 weeks | M | 8.53 ± 3.23 | 5.17 ± 0.47 | 8.80 ± 3.10 | 6.25 ± 0.39 | 2.31 ± 0.91 | 1.63 ± 0.02 | 2.31 ± 0.91 | 2.48 ± 1.22 |
| | F | 5.20 ± 0.58 | 3.99 ± 1.00 | 6.31 ± 1.08 | 3.45 ± 0.46 | 3.63 ± 1.57 | 2.85 ± 0.49 | 3.76 ± 1.41 | 3.92 ± 0.30 |
| 6 months | M | 5.20 ± 3.11 | 5.31 ± 2.61 | 4.80 ± .087 | 6.96 ± 3.50 | 5.36 ± 1.25 | 3.81 ± 0.84 | 5.65 ± 2.04 | 4.85 ± 0.58 |
| | F | 6.56 ± 1.98 | 4.55 ± 2.93 | 5.44 ± 0.90 | 5.63 ± 2.77 | 2.36 ± 2.45 | 4.12 ± 1.33 | 3.88 ± 0.49 | 5.24 ± 1.09 |
| 6 years | M | 4.15 ± 0.35 | 5.53 ± 0.84 | 4.65 ± 0.87 | 7.74 ± 2.13 | 4.69 ± 0.41 | 4.92 ± 1.11 | 4.94 ± 1.15 | 5.90 ± 0.60 |
| | F | 3.48 ± 0.32 | 4.65 ± 0.70 | 3.83 ± 0.06 | 4.80 ± 0.57 | 2.61 ± 0.80 | 3.69 ± 0.79 | 2.48 ± 0.56 | 5.47 ± 2.05 |
| Estrogen Treatment | | | | | | | | | |
| 1 day | M | 2.20 ± 0.63 | 3.71 ± 0.59 | | | 1.02 ± 0.29 | 0.85 ± 0.40 | | |
| | F | 2.65 ± 0.70 | 3.18 ± 0.63 | | | 1.24 ± 0.81 | 2.66 ± 0.01 | | |
| 6 weeks | M | 9.16 ± 4.63 | 8.21 ± 2.62 | 7.94 ± 2.10 | 9.16 ± 1.65 | 5.39 ± 1.90 | 2.78 ± 1.32 | 2.80 ± 2.60 | 4.13 ± 2.76 |
| | F | 5.48 ± 1.57 | 3.99 ± 0.36 | 10.83 ± 4.51 | 4.37 ± 0.26 | 4.73 ± 0.37 | 3.46 ± 0.84 | 1.50 ± 1.64 | 4.08 ± 0.44 |
| 6 months | M | 5.24 ± 0.81 | 4.50 ± 0.99 | 2.94 ± 0.27 | 4.76 ± 0.99 | 5.56 ± 0.25 | 4.88 ± 0.19 | 5.39 ± 0.47 | 6.14 ± 0.23 |
| | F | 5.25 ± 0.85 | 4.49 ± 1.73 | 5.63 ± 1.23 | 8.66 ± 5.49 | 4.82 ± 0.78 | 3.81 ± 1.90 | 4.59 ± 0.60 | 3.35 ± 1.88 |
| 6 years | M | 4.47 ± 0.55 | 5.57 ± 1.03 | 6.01 ± 3.62 | 7.22 ± 2.50 | 3.24 ± 1.08 | 4.62 ± 1.84 | 3.49 ± 1.91 | 5.70 ± 1.32 |
| | F | 4.03 ± 0.39 | 4.46 ± 0.53 | 5.23 ± 1.23 | 3.82 ± 0.57 | 3.70 ± 0.25 | 4.22 ± 0.95 | 3.42 ± 0.96 | 4.15 ± 0.47 |

Values expressed as ug protein/ug DNA ± SD.

M = male; F = female.

Table 2

Statistical Analysis of Protein Yields Presented in Table 1.

Age Comparisons

| | | "Lysosomal" Fraction | | | | Microsomal Fraction | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Thoracic | | Celiac | | Thoracic | | Celiac | |
| | | WC | SR | WC | SR | WC | SR | WC | SR |
| No Treatment | | | | | | | | | |
| 1 day - 6 weeks | M | S | N | | | S | N | | |
| | F | S | N | | | S | N | | |
| 6 weeks - 6 months | M | S | N | S | N | S | S | S | S |
| | F | N | N | N | N | N | N | N | N |
| 6 months - 6 years | M | N | N | N | N | N | N | N | N |
| | F | S | N | N | N | N | N | N | N |
| Estrogen Treatment | | | | | | | | | |
| 1 day - 6 weeks | M | S | S | | | S | S | | |
| | F | S | N | | | S | N | | |
| 6 weeks - 6 months | M | S | S | S | S | N | S | S | S |
| | F | N | N | S | S | N | N | S | N |
| 6 months - 6 years | M | N | N | S | N | S | N | S | N |
| | F | N | N | N | S | N | N | N | N |

S = significantly different ($P < 0.05$); N = not significantly different.

M = male; F = female.

Table 3

Statistical Analysis of Protein Yields Presented in Table 1.

Aortic Site Comparisons (Thoracic vs. Celiac)

| | | "Lysosomal" Fraction | | Microsomal Fraction | |
|---|---|---|---|---|---|
| | | WC | SR | WC | SR |
| No Treatment | | | | | |
| 6 weeks | M | N | N | N | N |
| | F | N | N | N | N |
| 6 months | M | N | N | N | N |
| | F | N | N | N | N |
| 6 years | M | N | N | N | N |
| | F | N | N | N | N |
| Estrogen Treatment | | | | | |
| 6 weeks | M | N | N | S | N |
| | F | S | N | S | N |
| 6 months | M | N | N | N | N |
| | F | N | S | N | N |
| 6 years | M | N | N | N | N |
| | F | N | N | N | N |

S = significantly different ($P < 0.05$); N = not significantly different.

M = male; F = female.

Table 4.

Statistical Analysis of Protein Yields Presented in Table 1.

Breed Comparisons (WC vs. SR)

| | | "Lysosomal" Fraction | | Microsomal Fraction | |
|---|---|---|---|---|---|
| | | Thoracic | Celiac | Thoracic | Celiac |
| No Treatment | | | | | |
| 1 day | M | N | | N | |
| | F | N | | N | |
| 6 weeks | M | S | N | N | N |
| | F | N | S | N | N |
| 6 months | M | N | N | N | N |
| | F | N | N | N | N |
| 6 years | M | N | S | N | N |
| | F | N | N | N | S |
| Estrogen Treatment | | | | | |
| 1 day | M | N | | N | |
| | F | N | | N | |
| 6 weeks | M | N | N | S | N |
| | F | N | S | N | S |
| 6 months | M | N | N | N | N |
| | F | N | S | N | N |
| 6 years | M | N | N | N | S |
| | F | N | N | N | N |

S = significantly different ($P < 0.05$); N = not significantly different.

M = male; F = female.

Table 5

Statistical Analysis of Protein Yields Presented in Table 1.

Sex Comparisons (M vs. F)

| | "Lysosomal" Fraction | | | | Microsomal Fraction | | | |
|---|---|---|---|---|---|---|---|---|
| | Thoracic | | Celiac | | Thoracic | | Celiac | |
| | WC | SR | WC | SR | WC | SR | WC | SR |
| No Treatment | | | | | | | | |
| 1 day | N | N | | | N | N | | |
| 6 weeks | N | N | N | S | N | N | N | N |
| 6 months | N | N | N | N | S | N | N | N |
| 6 years | N | N | N | S | S | N | S | N |
| Estrogen Treatment | | | | | | | | |
| 1 day | N | N | | | N | S | | |
| 6 weeks | N | S | S | S | N | N | N | N |
| 6 months | N | N | N | S | N | N | N | S |
| 6 years | N | N | N | S | N | N | N | N |

S = significantly different ($P < 0.05$); N = not significantly different.

Table 6

Statistical Analysis of Protein Yields Presented in Table 1.

Treatment Comparisons (Control vs. Estrogen)

| | | "Lysosomal" Fraction | | | | Micrososomal Fraction | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Thoracic | | Celiac | | Thoracic | | Celiac | |
| | | WC | SR | WC | SR | WC | SR | WC | SR |
| 1 day | M | N | N | | | N | N | | |
| | F | N | N | | | N | N | | |
| 6 weeks | M | N | S | N | S | S | N | N | N |
| | F | N | N | S | N | N | N | S | N |
| 6 months | M | N | N | N | N | N | N | N | S |
| | F | S | N | N | S | S | N | N | N |
| 6 years | M | N | N | N | N | N | N | N | N |
| | F | N | N | N | N | N | N | N | N |

S = significantly different ($P < 0.05$); N = not significantly different.

Table 7.

N-Acetyl-B-Hexosaminidase Activity in Sub-cellular Fractions From the Upper Thoracic Aorta of White Carneau (WC) and Show Racer (SR) Pigeons as a Function of Age and Brij-35 Treatment.

| | | Homogenate | | "Lysosomal" Fraction | | Microsomal Fraction | | Soluble Fraction | |
|---|---|---|---|---|---|---|---|---|---|
| | | WC | SR | WC | SR | WC | SR | WC | SR |
| No treatment | | | | | | | | | |
| 1 day | Brij-35 | 80 ± 11 | 142 ± 34 | 33 ± 6.1 | 55 ± 27 | 3.2 ± 2.0 | 5.5 ± 2.3 | 27 ± 4.9 | 48 ± 16 |
| | Free | 68 ± 6.1 | 124 ± 31 | 25 ± 5.4 | 42 ± 18 | 2.6 ± 1.7 | 5.8 ± 3.2 | 30 ± 3.5 | 48 ± 1.2 |
| 6 weeks | Brij-35 | 58 ± 17 | 41 ± 6.1 | 22 ± 8.6 | 15 ± 3.0 | 3.4 ± 2.3 | 3.3 ± 1.5 | 20 ± 9.2 | 15 ± 6.8 |
| | Free | 55 ± 16 | 39 ± 7.9 | 17 ± 3.7 | 9.8 ± 2.2 | 3.1 ± 2.0 | 2.5 ± 0.4 | 26 ± 11 | 23 ± 9.3 |
| 6 months | Brij-35 | 29 ± 11 | 31 ± 9.4 | 11 ± 4.3 | 13 ± 3.8 | 3.8 ± 4.0 | 3.0 ± 0.5 | 7.0 ± 2.5 | 9.2 ± 3.5 |
| | Free | 25 ± 11 | 25 ± 6.9 | 6.5 ± 2.7 | 8.9 ± 3.4 | 1.7 ± 0.5 | 3.0 ± 3.2 | 11.2 ± 5.2 | 12 ± 4.6 |
| 6 years | Brij-35 | 20 ± 3.9 | 28 ± 2.8 | 8.0 ± 2.2 | 10 ± 1.4 | 2.7 ± 2.4 | 2.0 ± 1.1 | 6.6 ± 2.0 | 8.1 ± 2.2 |
| | Free | 17 ± 4.1 | 24 ± 5.0 | 5.5 ± 1.7 | 7.1 ± 1.6 | 1.6 ± 0.6 | 2.5 ± 1.1 | 9.2 ± 1.4 | 12 ± 1.9 |
| Estrogen Treatment | | | | | | | | | |
| 1 day | Brij-35 | 97 ± 16 | 142 ± 30 | 39 ± 7.5 | 47 ± 6.9 | 5.3 ± 1.6 | 8.7 ± 3.6 | 23 ± 7.4 | 50 ± 16 |
| | Free | 78 ± 16 | 125 ± 35 | 28 ± 4.9 | 37 ± 4.4 | 4.0 ± 1.3 | 6.6 ± 3.6 | 25 ± 8.6 | 57 ± 23 |
| 6 weeks | Brij-35 | 61 ± 27 | 43 ± 8.3 | 18 ± 5.6 | 19 ± 5.2 | 5.6 ± 2.9 | 3.4 ± 2.5 | 22 ± 14 | 12 ± 5.7 |
| | Free | 64 ± 26 | 39 ± 7.1 | 16 ± 3.3 | 12 ± 4.7 | 4.8 ± 3.2 | 2.4 ± 1.4 | 33 ± 2.0 | 22 ± 7.7 |
| 6 months | Brij-35 | 29 ± 9.8 | 30 ± 4.6 | 11 ± 2.4 | 12 ± 3.7 | 2.3 ± 1.3 | 2.9 ± 0.9 | 7.7 ± 1.9 | 8.1 ± 2.0 |
| | Free | 24 ± 8.7 | 28 ± 3.5 | 6.0 ± 2.5 | 8.5 ± 2.3 | 2.6 ± 1.4 | 2.2 ± 0.9 | 11 ± 2.9 | 11 ± 2.6 |
| 6 years | Brij-35 | 23 ± 3.1 | 26 ± 4.4 | 9.0 ± 1.9 | 8.9 ± 1.5 | 1.8 ± 0.8 | 1.5 ± 1.1 | 9.4 ± 1.7 | 6.9 ± 0.9 |
| | Free | 20 ± 2.8 | 23 ± 3.8 | 5.5 ± 1.3 | 7.0 ± 1.7 | 1.5 ± 1.6 | 2.8 ± 1.9 | 11 ± 2.4 | 11 ± 1.2 |

Values expressed as ng substrate liberated/min./ug DNA ± SD.

Table 8.

N-Acetyl-B-Hexosominidase Activity in Sub-cellular Fractions from the Celiac Bifurcation of White Carneau (WC) and Show Racer (SR) Aortas as a Function of Age and Brij-35 Treatment

| | | Homogenate | | "Lysosomal" Fraction | | Microsomal Fraction | | Soluble Fraction | |
|---|---|---|---|---|---|---|---|---|---|
| | | WC | SR | WC | SR | WC | SR | WC | SR |
| No treatment | | | | | | | | | |
| 6 weeks | Brij-35 | 59 ± 12 | 35 ± 6.1 | 21 ± 5.5 | 14 ± 2.4 | 4.0 ± 2.7 | 4.1 ± 0.7 | 20.0 ± 6.0 | 11.3 ± 4.5 |
| | Free | 57 ± 8.4 | 30 ± 4.7 | 18 ± 4.3 | 9.4 ± 1.4 | 2.6 ± 0.8 | 3.0 ± 1.4 | 24.8 ± 8.2 | 15.8 ± 4.6 |
| 6 months | Brij-35 | 30 ± 8.5 | 28 ± 4.0 | 10 ± 2.3 | 12 ± 3.8 | 2.6 ± 1.3 | 2.8 ± 0.8 | 8.3 ± 2.2 | 8.1 ± 1.8 |
| | Free | 25 ± 4.8 | 26 ± 4.6 | 6.4 ± 1.8 | 7.8 ± 1.9 | 2.0 ± 1.1 | 2.1 ± 0.3 | 10.1 ± 3.2 | 10.2 ± 2.4 |
| 6 years | Brij-35 | 35 ± 13 | 23 ± 4.5 | 11 ± 2.8 | 9.7 ± 1.5 | 3.7 ± 2.1 | 1.4 ± 1.1 | 16.4 ± 6.6 | 6.6 ± 2.7 |
| | Free | 32 ± 10 | 23 ± 5.5 | 6.3 ± 0.7 | 6.1 ± 0.7 | 1.9 ± 0.9 | 1.8 ± 1.2 | 20.9 ± 7.8 | 10.0 ± 2.1 |
| Estrogen treatment | | | | | | | | | |
| 6 weeks | Brij-35 | 72 ± 30 | 43 ± 17 | 25 ± 5.6 | 19 ± 7.9 | 2.4 ± 0.7 | 4.2 ± 2.9 | 24 ± 7.6 | 13.8 ± 8.9 |
| | Free | 68 ± 27 | 36 ± 13 | 21 ± 5.9 | 13 ± 4.3 | 2.1 ± 1.3 | 5.1 ± 1.4 | 26.8 ± 8.2 | 17.6 ± 6.7 |
| 6 months | Brij-35 | 33 ± 7.2 | 32 ± 6.4 | 12 ± 3.8 | 9.9 ± 4.5 | 4.1 ± 1.6 | 4.1 ± 0.6 | 7.4 ± 1.8 | 10.8 ± 2.6 |
| | Free | 28 ± 6.5 | 30 ± 3.2 | 7.1 ± 3.2 | 9.3 ± 1.9 | 3.2 ± 1.1 | 2.0 ± 1.1 | 11.2 ± 23 | 13.5 ± 3.2 |
| 6 years | Brij-35 | 39 ± 13 | 21 ± 3.5 | 13 ± 3.0 | 8.6 ± 0.6 | 4.0 ± 2.8 | 1.3 ± 0.4 | 15.3 ± 4.9 | 7.2 ± 2.2 |
| | Free | 35 ± 12 | 19 ± 4.4 | 7.8 ± 2.2 | 6.3 ± 1.0 | 2.1 ± 1.8 | 1.9 ± 0.8 | 24.1 ± 10 | 8.9 ± 1.2 |

Values expressed as ng substrate liberated/min./ug DNA ± SD.

Table 9.

Statistical Analysis of N-Acetyl-B-Hexosominidase Activities Presented in Tables 7 and 8.

Age Comparisons

| Thoracic | | Homogenate | | "Lysosomal" Fraction | | Microsomal Fraction | | Soluble Fraction | |
|---|---|---|---|---|---|---|---|---|---|
| | | WC | SR | WC | SR | WC | SR | WC | SR |
| 1 day - 6 weeks | Brij-35 | S | S | S | S | N | S | N | S |
| | Free | N | S | S | S | N | S | N | S |
| 6 weeks - 6 months | Brij-35 | S | N | N | N | N | N | S | N |
| | Free | S | N | S | N | N | N | S | S |
| 6 months - 6 years | Brij-35 | N | N | N | N | N | N | N | N |
| | Free | N | N | N | N | N | N | N | N |
| Celiac | | | | | | | | | |
| 6 weeks - 6 months | Brij-35 | S | N | S | N | N | N | S | N |
| | Free | S | N | S | N | N | N | S | N |
| 6 months - 6 years | Brij-35 | N | N | N | N | N | N | N | N |
| | Free | N | N | N | N | N | N | S | N |

S = significantly different ($P < 0.05$); N = not significantly different.

Table 10.

Statistical Analysis of N-Acetyl-B-Hexosominidase Activities Presented in Tables 7 and 8.
Breed Comparisons (WC vs. SR)

| No treatment | | Homogenate | | "Lysosomal" Fraction | | Microsomal Fraction | | Soluble Fraction | |
|---|---|---|---|---|---|---|---|---|---|
| | | Thoracic | Celiac | Thoracic | Celiac | Thoracic | Celiac | Thoracic | Celiac |
| 1 day | Brij-35 | S | | S | | S | | S | |
| | Free | S | | S | | S | | S | |
| 6 weeks | Brij-35 | N | S | N | N | N | N | N | S |
| | Free | N | S | N | S | N | N | N | N |
| 6 months | Brij-35 | N | N | N | N | N | N | N | N |
| | Free | N | N | N | N | N | N | N | N |
| 6 years | Brij-35 | N | N | N | N | N | S | N | S |
| | Free | N | N | N | N | N | N | N | S |
| Estrogen Treatment | | | | | | | | | |
| 1 day | Brij-35 | S | | N | | S | | S | |
| | Free | S | | S | | S | | S | |
| 6 weeks | Brij-35 | N | S | N | N | N | N | S | S |
| | Free | S | S | N | S | N | S | S | N |
| 6 months | Brij-35 | N | N | N | N | N | N | N | N |
| | Free | N | N | N | N | N | N | N | N |
| 6 years | Brij-35 | N | N | N | N | N | S | N | N |
| | Free | N | N | N | N | N | N | N | S |

S = significantly different ($P < 0.05$); N = not significantly different.

Table 11.

Statistical Analysis of N-Acetyl-B-Hexosaminidase Activities Following Estrogen Treatment Presented in Tables 7 and 8. Age Comparisons.

| | | Homogenate | | "Lysosomal" Fraction | | Microsomal Fraction | | Soluble Fraction | |
|---|---|---|---|---|---|---|---|---|---|
| Thoracic | | WC | SR | WC | SR | WC | SR | WC | SR |
| 1 day - 6 weeks | Brij-35 | S | S | S | S | N | S | N | S |
| | Free | N | S | S | S | N | S | N | S |
| 6 weeks - 6 months | Brij-35 | S | N | N | N | N | N | S | N |
| | Free | S | N | S | N | N | N | S | S |
| 6 months - 6 years | Brij-35 | N | N | N | N | N | N | N | N |
| | Free | N | N | N | N | N | N | N | N |
| Celiac | | | | | | | | | |
| 6 weeks - 6 months | Brij-35 | S | N | S | N | N | N | S | N |
| | Free | S | N | S | N | N | S | S | N |
| 6 months - 6 years | Brij-35 | N | N | N | N | N | S | N | N |
| | Free | N | N | N | N | N | N | S | N |

S = significantly different ($P < 0.05$); N = not significantly different.

Table 12.

Decrease in N-Acetyl-B-Hexosaminidase Activity in the Soluble Fraction After Brij-35 Treatment.

| | | Thoracic WC Control | Estrogen | SR Control | Estrogen | Celiac WC Control | Estrogen | SR Control | Estrogen |
|---|---|---|---|---|---|---|---|---|---|
| 1 day | Actual | 2.9 ± 6.0 | 3.8 ± 4.7 | 0.0* | 6.7 ± 8.0 | | | | |
| | % | 9.7 ± 11.8* | 9.1 ± 9.7 | 0.0 + 0.2 | 11.9 ± 8.3 | | | | |
| 6 weeks | Actual | 6.0 ± 4.9 | 11.5 ± 11.1 | 8.2 ± 5.0 | 9.9 ± 7.4 | 5.2 ± 5.2 | 3.2 ± 6.0* | 4.5 ± 1.8 | 3.8 ± 4.0 |
| | % | 23.1 ± 16.0 | 34.3 ± 18.2 | 35.3 ± 15.8 | 44.6 ± 22.4 | 19.4 ± I6.4 | 10.4 ± 14.9 | 28.5 ± 12.6 | 21.6 ± 18.2 |
| 6 months | Actual | 4.2 ± 3.7 | 2.8 ± 1.2 | 2.6 ± 2.1 | 3.2 ± 1.2 | 1.8 ± 2.9 | 3.8 ± 1.7 | 2.1 ± 2.6 | 1.7 ± 2.9 |
| | % | 37.5 ± 17.6 | 26.7 ± 5.9 | 22.0 ± 14.1 | 28.3 ± 8.9 | 17.8 ± 17.6 | 33.9 ± 9.8 | 20.6 ± 17.3 | 20.0 ± 15.3 |
| 6 years | Actual | 2.6 ± 1.2 | 2.0 ± 0.9 | 3.8 ± 1.0 | 4.3 ± 1.3 | 4.5 ± 4.5 | 8.8 ± 7.7 | 3.5 ± 0.8 | 1.7 ± 1.7 |
| | % | 29.5 ± 21.2 | 17.5 ± 5.4 | 31.9 ± 9.7 | 38.4 ± 19.2 | 22.8 ± 18.8 | 36.5 ± 17.9 | 36.2 ± 14.8 | 19.1 ± 19.7 |

Actual = actual difference between Brij-35 activity and Free activity in ng/min./ug DNA.

% = % decrease in activity

* Not significantly different from 0 ($P < 0.05$).

Table 13.

Statistical Analysis of the Decrease in N-Acetyl-B-Hexosaminidase Activity Presented in Table 12.

Breed Comparisons (WC vs. SR)

| | | Thoracic | | Celiac | |
|---|---|---|---|---|---|
| | | Control | Estrogen | Control | Estrogen |
| 1 day | Actual | N | N | | |
| | % | N | N | | |
| 6 weeks | Actual | N | N | N | N |
| | % | N | N | N | N |
| 6 months | Actual | N | N | N | N |
| | % | N | N | N | N |
| 6 years | Actual | N | N | N | N |
| | % | N | S | S | N |

S = significantly different ($P < 0.05$); N = not significantly different.

Table 14.

Statistical Analysis of Decreases in N-Acetyl-B-Hexosaminidase Activity Presented in Table 12.

Aortic Site Comparisons (Thoracic vs. Celiac)

| | | WC | | SR | |
|---|---|---|---|---|---|
| | | Control | Estrogen | Control | Estrogen |
| 6 weeks | Actual | N | N | N | N |
| | % | N | S | N | N |
| 6 months | Actual | N | N | N | N |
| | % | N | N | N | N |
| 6 years | Actual | N | N | N | S |
| | % | N | S | N | N |

S = significantly different ($P<0.05$); N = not significantly different.

Table 15.

Percentage of Latent N-Acetyl-B-Hexosaminidase Activity in the "Lysosomal" Fraction.

| | | Upper Thoracic | | | | Celiac Bifurcation | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | WC | | SR | | WC | | SR | |
| | | Control | Estrogen | Control | Estrogen | Control | Estrogen | Control | Estrogen |
| 1 day | Actual | 8 ± 9 | 12 ± 4 | 13 ± 10 | 10 ± 3 | | | | |
| | % | 24 ± 8 | 28 ± 13 | 24 ± 9 | 21 ± 4 | | | | |
| 6 weeks | Actual | 5.7 ± 7.8* | 2 ± 3* | 5.2 ± 1.3 | 6.7 ± 2.5 | 3.0 ± 2.9* | 4.0 ± 2.0 | 4.6 ± 1.6 | 6.0 ± 4.2 |
| | % | 23 ± 15 | 11 ± 12 | 33 ± 4 | 36 ± 13 | 14 ± 10 | 16 ± 13 | 33 ± 7 | 32 ± 15 |
| 6 months | Actual | 5.2 ± 3.0 | 4.6 ± 2.6 | 3.9 ± 2.0 | 3.5 ± 1.9 | 3.8 ± 3.4 | 4.9 ± 1.9 | 4.2 ± 1.6 | 0.7 ± 0.7* |
| | % | 45 ± 19 | 45 ± 24 | 32 ± 19 | 29 ± 12 | 36 ± 23 | 41 ± 18 | 35 ± 12 | 7 ± 7 |
| 6 years | Actual | 2.5 ± 0.7 | 3.5 ± 1.7 | 3.2 ± 1.7 | 2.0 ± 2.8* | 4.4 ± 1.9 | 5.4 ± 1.0 | 3.9 ± 1.9 | 2.7 ± 0.9 |
| | % | 38 ± 5 | 33 ± 13 | 29 ± 16 | 22 ± 22 | 43 ± 10 | 40 ± 5 | 39 ± 8 | 30 ± 10 |

$$\% \text{ Latency} = \frac{\text{Brij-35 activity} - \text{Free activity}}{\text{Brij-35 activity}} \times 100$$

Actual = actual difference between Brij-35 activity and the Free activity in ng/min./ug DNA.

% = % Latency

* Not significantly different from 0 ($P < 0.05$).

Table 16.

Statistical Analysis of Latent N-Acetyl-B-hexosaminidase Activities Presented in Table

Breed (WC vs. SR) and Aortic Site (Thoracic vs. Celiac) Comparisons

| | | Breed Comparisons | | | | Aortic Site Comparisons | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Upper Thoracic | | Celiac Bifurcation | | WC | | SR | |
| | | Control | Estrogen | Control | Estrogen | Control | Estrogen | Control | Estrogen |
| 1 day | Actual | N | N | | | | | | |
| | % | N | N | | | | | | |
| 6 weeks | Actual | N | S | N | N | N | N | N | N |
| | % | N | S | S | N | N | N | N | N |
| 6 months | Actual | N | N | N | S | N | N | N | S |
| | % | N | N | N | S | N | N | N | S |
| 6 years | Actual | N | N | N | S | N | N | N | N |
| | % | N | N | N | N | N | N | N | N |

S = significantly different ($P < 0.05$); N = not significantly different.

Table 17.

**Statistical Analysis of Latent N-Acetyl-B-Hexosaminidase Activities Presented in Table 15.**

**Age Comparisons**

| | Upper Thoracic | | | | Celiac Bifurcation | | | |
|---|---|---|---|---|---|---|---|---|
| | WC | | SR | | WC | | SR | |
| | Control | Estrogen | Control | Estrogen | Control | Estrogen | Control | Estrogen |
| 1 day-6 weeks | | | | | | | | |
| Actual | N | N | N | N | | | | |
| % | N | N | N | N | | | | |
| 6 weeks-6 months | | | | | | | | |
| Actual | N | N | N | N | N | N | N | S |
| % | N | S | N | N | N | S | N | S |
| 6 months - 6 years | | | | | | | | |
| Actual | N | N | N | N | N | N | N | N |
| % | N | N | N | N | N | N | N | S |
| 1 day-6 months | | | | | | | | |
| Actual | N | S | N | N | | | | |
| % | S | N | N | N | | | | |
| 1 day-6 years | | | | | | | | |
| Actual | S | S | S | S | | | | |
| % | S | N | N | N | | | | |
| 6 weeks-6 years | | | | | | | | |
| Actual | N | N | N | N | N | N | N | N |
| % | N | N | N | N | S | S | N | N |

S = significantly different ($P < 0.05$); N = not significantly different.

Table 18.

Acid Phosphatase Activity in Sub-cellular Fractions From the Upper Thoracic Aorta of White Carneau (WC) and Show Racer (SR) Pigeons as a Function of Age, Sex and Brij-35 Treatment

| | | | Homogenate | | "Lysosomal" Fraction | | Microsomal Fraction | | Soluble Fraction | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | WC | SR | WC | SR | WC | SR | WC | SR |
| 1 day | Brij-35 | M | 97.5 ± 8.6 | 191 ± 28.0 | 54.9 ± 4.6 | 99.7 ± 20.4 | 6.3 ± 1.8 | 19.3 ± 5.2 | 19.0 ± 1.4 | 36.3 ± 8.8 |
| | | F | 109 ± 8.1 | 139 ± 28.8 | 51.7 ± 26.3 | 73.8 ± 5.7 | 16.2 ± 8.0 | 18.9 ± 15.9 | 12.6 ± 12.1 | 13.5 ± 1.0 |
| | Free | M | 84.1 ± 5.5 | 153 ± 33.1 | 48.5 ± 4.5 | 87.0 ± 13.0 | 4.6 ± 0.1 | 21.0 ± 5.6 | 19.4 ± 1.1 | 36.3 ± 5.4 |
| | | F | 83.6 ± 2.7 | 123 ± 22 | 42.6 ± 20.2 | 68.0 ± 4.5 | 17.0 ± 6.0 | 25.7 ± 17.8 | 12.8 ± 10.9 | 12.5 ± 5.4 |
| 6 weeks | Brij-35 | M | 114 ± 0.5 | 114 ± 8.5 | 70.0 ± 13.0 | 74.0 ± 7.0 | 6.9 ± 3.1 | 3.9 ± 4.0 | 4.9 ± 2.3 | 14.4 ± 10.6 |
| | | F | 121 ± 51.5 | 83.6± 5.5 | 77.3 ± 31.5 | 49.2 ± 11.2 | 8.9 ± 9.0 | 7.6 ± 0.4 | 5.4 ± 4.8 | 2.4 ± 3.6 |
| | Free | M | 113 ± 15.0 | 117 ± 9.0 | 65.5 ± 17.5 | 61.5 ± 3.5 | 8.2 ± 4.8 | 5.5 ± 5.5 | 15.5 ± 6.5 | 20.0 ± 8.0 |
| | | F | 98.7 ± 26.5 | 73.2± 13.1 | 54.3 ± 23.5 | 41.0 ± 9.6 | 9.0 ± 7.9 | 10.0 ± 1.7 | 9.8 ± 2.9 | 4.7 ± 4.7 |
| 6 months | Brij-35 | M | 159 ± 31.7 | 127 ± 22.9 | 83.1 ± 23.5 | 81.0 ± 35.8 | 16.1 ± 8.0 | 9.9 ± 2.1 | 2.3 ± 3.9 | 6.8 ± 5.3 |
| | | F | 108 ± 64.5 | 89.7± 15.6 | 60.3 ± 43.0 | 57.7 ± 24.6 | 5.7 ± 3.9 | 12.1 ± 6.9 | 2.6 ± 0.4 | 2.4 ± 2.2 |
| | Free | M | 123 ± 8.5 | 99.7± 10.1 | 69.4 ± 21.5 | 66.7 ± 31.5 | 16.6 ± 5.2 | 8.9 ± 3.6 | 7.1 ± 8.1 | 4.3 ± 6.6 |
| | | F | 82.2 ± 37.7 | 76.7± 17.9 | 54.1 ± 32.2 | 39.3 ± 15.9 | 4.8 ± 3.8 | 15.1 ± 6.8 | 5.6 ± 1.2 | 3.6 ± 1.5 |
| 6 years | Brij-35 | M | 112 ± 10.0 | 125 ± 5.7 | 77.9 ± 8.2 | 73.4 ± 22.7 | 13.8 ± 1.4 | 17.9 ± 0.8 | 2.1 ± 1.2 | 0.5 ± 0.9 |
| | | F | 88.2 ± 13.8 | 83.0± 19.3 | 49.1 ± 6.7 | 59.5 ± 16.9 | 7.6 ± 2.9 | 9.3 ± 5.5 | 1.3 ± 2.5 | 0.0 ± 0.0 |
| | Free | M | 107 ± 12.1 | 112 ± 14.5 | 60.6 ± 3.4 | 67.2 ± 9.0 | 19.7 ± 8.5 | 16.9 ± 5.4 | 7.9 ± 9.4 | 16.5 ± 9.0 |
| | | F | 67.3 ± 1.4 | 87.0± 15.0 | 37.6 ± 3.4 | 48.8 ± 12.0 | 9.1 ± 2.7 | 9.0 ± 3.2 | 6.3 ± 3.6 | 6.5 ± 1.4 |

Values expressed as ng substrate liberated/min./ug DNA ± SD.

M = male; F = female.

Table 19.

Acid Phosphatase Activity Following Estrogen Treatment in Sub-cellular Fractions From the Upper Thoracic Aorta of White Carneau (WC) and Show Racer (SR) Pigeons as a Function of Age, Sex, and Brij-35 Treatment

| | | | Homogenate | | "Lysosomal" Fraction | | Microsomal Fraction | | Soluble Fraction | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | WC | SR | WC | SR | WC | SR | WC | SR |
| 1 day | Brij-35 | M | 129 ± 12.3 | 230 ± 88.2 | 61.7 ± 11.6 | 109.4 ± 43.8 | 25.8 ± 0.4 | 33.5 ± 3.9 | 13.4 ± 11.4 | 63.0 ± 35.4 |
| | | F | 133 ± 14.6 | 148 ± 10.4 | 71.3 ± 6.8 | 57.9 ± 6.5 | 21.3 ± 14.1 | 45.3 ± 1.1 | 10.9 ± 12.7 | 8.1 ± 2.1 |
| | Free | M | 104 ± 3.7 | 199 ± 83.8 | 43.6 ± 8.7 | 92.6 ± 44 | 21.6 ± 1.1 | 25.2 ± 2.5 | 12.2 ± 10.8 | 57.3 ± 34.4 |
| | | F | 101 ± 1.6 | 109 ± 4.9 | 57.8 ± 4.4 | 46.8 ± 2.9 | 20.0 ± 14.2 | 37.9 ± 5.5 | 10.5 ± 10.9 | 7.7 ± 4.8 |
| 6 weeks | Brij-35 | M | 80.0 ± 24.0 | 144 ± 16.5 | 71.0 ± 13.0 | 100.0 ± 19.0 | 10.6 ± 3.5 | 1.3 ± 1.3 | 1.3 ± 0.1 | 5.5 ± 5.5 |
| | | F | 103 ± 41.3 | 83.6 ± 9.9 | 70.0 ± 31.5 | 46.6 ± 9.7 | 14.3 ± 2.1 | 0.4 ± 3.6 | 4.3 ± 7.5 | 3.0 ± 4.2 |
| | Free | M | 85.0 ± 11.0 | 114 ± 14.5 | 67.5 ± 4.5 | 79.5 ± 14.5 | 9.7 ± 3.3 | 1.7 ± 1.7 | 5.7 ± 3.0 | 10.0 ± 10.0 |
| | | F | 96.8 ± 20.9 | 71.4 ± 7.2 | 59.0 ± 20.8 | 43.3 ± 7.8 | 11.1 ± 2.8 | 8.9 ± 3.6 | 8.9 ± 12.0 | 4.2 ± 4.0 |
| 6 months | Brij-35 | M | 157 ± 30.1 | 97.7 ± 12.0 | 93.2 ± 15.1 | 68.0 ± 4.0 | 16.2 ± 4.8 | 12.0 ± 5.8 | 4.7 ± 5.0 | 2.0 ± 0.8 |
| | | F | 98.5 ± 31.3 | 97.0 ± 14.2 | 50.4 ± 25.4 | 55.7 ± 12.4 | 2.9 ± 2.6 | 14.9 ± 15.7 | 1.0 ± 1.3 | 0.0 ± 0.0 |
| | Free | M | 129 ± 21.1 | 85.9 ± 6.9 | 73.6 ± 6.6 | 52.0 ± 6.1 | 15.9 ± 7.7 | 14.1 ± 5.7 | 10.9 ± 7.6 | 2.1 ± 0.9 |
| | | F | 74.2 ± 18.1 | 78.3 ± 12.6 | 45.3 ± 13.6 | 44.0 ± 13.1 | 4.8 ± 4.2 | 11.6 ± 9.9 | 1.2 ± 1.5 | 1.5 ± 1.6 |
| 6 years | Brij-35 | M | 147 ± 21.1 | 115 ± 12.1 | 87.9 ± 9.9 | 78.5 ± 5.5 | 13.0 ± 6.6 | 20.8 ± 12.1 | 5.7 ± 4.3 | 0.0 ± 0.0 |
| | | F | 113 ± 15.3 | 98.6 ± 14.7 | 67.7 ± 11.3 | 55.8 ± 3.9 | 14.7 ± 4.1 | 11.9 ± 8.2 | 12.4 ± 21.4 | 3.4 ± 3.5 |
| | Free | M | 111 ± 13.8 | 99.6 ± 14.5 | 68.3 ± 5.0 | 64.0 ± 3.7 | 15.9 ± 4.2 | 21.7 ± 10.6 | 10.3 ± 7.6 | 4.0 ± 4.1 |
| | | F | 85.8 ± 2.4 | 88.3 ± 28.8 | 48.5 ± 13.1 | 46.7 ± 3.6 | 12.6 ± 3.2 | 14.9 ± 4.5 | 1.7 ± 2.1 | 5.6 ± 3.8 |

Values expressed as ng substrate liberated/min./ug DNA ± SD.

M = male; F = female.

Table 20.

Acid Phosphatase Activity in Sub-cellular Fractions From the Celiac Bifurcation of White Carneau (WC) and Show Racer (SR) Aortas as a Function of Age, Sex, and Brij-35 Treatment

| | | | Homogenate | | "Lysosomal" Fraction | | Microsomal Fraction | | Soluble Fraction | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | WC | SR | WC | SR | WC | SR | WC | SR |
| 6 weeks | Brij-35 | M | 148 ± 7.0 | 112 ± 14.5 | 90.0 ± 1.0 | 76.5 ± 4.5 | 6.5 ± 0.4 | 20.3 ± 4.8 | 24.8 ± 22.2 | 4.4 ± 3.2 |
| | | F | 122 ± 27.0 | 90 ± 17 | 86.0 ± 19.5 | 42.0 ± 5.2 | 17.2 ± 12.0 | 17.6 ± 7.0 | 7.0 ± 12.0 | 4.4 ± 5.0 |
| | Free | M | 136 ± 2.0 | 93.0 ± 8.0 | 88.0 ± 3.0 | 58.0 ± 2.0 | 8.8 ± 0.5 | 9.5 ± 9.5 | 19.3 ± 16.7 | 6.8 ± 6.2 |
| | | F | 139 ± 48.5 | 73.6 ± 13.4 | 56.3 ± 18.3 | 35.3 ± 2.1 | 15.1 ± 7.8 | 19.7 ± 6.7 | 8.8 ± 10.2 | 5.0 ± 6.1 |
| 6 months | Brij-35 | M | 167 ± 31.6 | 142 ± 72.4 | 97.3 ± 17.0 | 96.1 ± 55.5 | 40.1 ± 25.8 | 22.1 ± 8.4 | 5.4 ± 9.2 | 4.2 ± 3.5 |
| | | F | 85.2 ± 28.1 | 95.7 ± 17.0 | 50.3 ± 12.4 | 60.8 ± 24.7 | 6.4 ± 6.5 | 17.3 ± 7.6 | 1.2 ± 1.7 | 0.4 ± 0.6 |
| | Free | M | 115 ± 16.4 | 121 ± 44.1 | 72.9 ± 16.9 | 81.0 ± 18.3 | 26.5 ± 10.3 | 24.1 ± 11.5 | 7.6 ± 10.8 | 3.9 ± 5.4 |
| | | F | 68.5 ± 14.7 | 73.9 ± 9.6 | 46.0 ± 14.2 | 44.9 ± 13.5 | 5.8 ± 6.3 | 13.3 ± 6.8 | 0.9 ± 0.9 | 4.1 ± 5.8 |
| 6 years | Brij-35 | M | 208 ± 15.2 | 139 ± 24.4 | 111 ± 17.4 | 98.9 ± 4.1 | 47.5 ± 15.2 | 23.4 ± 5.6 | 2.5 ± 2.2 | 2.2 ± 1.9 |
| | | F | 159 ± 9.9 | 107 ± 25.9 | 90.4 ± 16.3 | 61.7 ± 9.0 | 17.2 ± 11.2 | 20.8 ± 14.3 | 18.2 ± 9.0 | .07 ± 0.11 |
| | Free | M | 166 ± 18.5 | 117 ± 13.0 | 84.3 ± 17.6 | 81.4 ± 7.0 | 48.1 ± 19.7 | 23.8 ± 7.1 | 8.0 ± 6.1 | 2.3 ± 1.3 |
| | | F | 128 ± 18.7 | 79.1 ± 11.7 | 66.8 ± 10.5 | 52.3 ± 0.8 | 14.4 ± 8.0 | 15.6 ± 15.5 | 24.5 ± 15.8 | 6.0 ± 5.2 |

Values expressed as ng substrate liberated/min./ug DNA ± SD.

M = male; F = female.

Table 21.

Acid Phosphatase Activity Following Estrogen Treatment in Sub-cellular Fractions from the Celiac Bifurcation of White Carneau (WC) and Show Racer (SR) Aortas as a Function of Age, Sex, and Brij-35 Treatment

| | | | Homogenate | | "Lysosomal" Fraction | | Microsomal Fraction | | Soluble Fraction | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | WC | SR | WC | SR | WC | SR | WC | SR |
| 6 weeks | Brij-35 | M | 161 ± 10.0 | 204 ± 80.0 | 120 ± 0.1 | 123 ± 51.5 | 21.6 ± 21.4 | 18.0 ± 9.1 | 7.4 ± 3.7 | 3.9 ± 3.9 |
| | | F | 152 ± 66.7 | 102 ± 28.0 | 118 ± 49.8 | 58.2 ± 2.9 | 10.3 ± 10.1 | 14.2 ± 1.0 | 5.7 ± 9.8 | 2.6 ± 3.1 |
| | Free | M | 137 ± 13.0 | 159 ± 68.0 | 97.0 ± 4.0 | 95.5 ± 34.5 | 19.2 ± 12.9 | 17.5 ± 8.6 | 7.0 ± 5.1 | 4.0 ± 4.0 |
| | | F | 119 ± 45.0 | 73.4 ± 18.2 | 86.7 ± 40.6 | 43.3 ± 2.5 | 3.9 ± 4.2 | 17.2 ± 5.9 | 6.0 ± 10.4 | 3.3 ± 3.2 |
| 6 months | Brij-35 | M | 156 ± 2.9 | 128 ± 35.0 | 70.9 ± 8.6 | 68.4 ± 5.4 | 45.6 ± 19.3 | 32.8 ± 9.8 | 0.7 ± 0.6 | 4.2 ± 2.5 |
| | | F | 92.7 ± 23.3 | 130 ± 21.0 | 55.8 ± 17.9 | 92.0 ± 32.5 | 12.7 ± 7.1 | 13.3 ± 2.5 | 1.5 ± 1.4 | 4.3 ± 7.5 |
| | Free | M | 116 ± 14.2 | 102 ± 9.2 | 46.0 ± 12.9 | 49.1 ± 4.1 | 41.1 ± 13.2 | 28.6 ± 9.6 | 3.2 ± 5.6 | 2.8 ± 2.1 |
| | | F | 72.7 ± 12.7 | 93.7 ± 8.3 | 52.5 ± 13.3 | 66.3 ± 25.5 | 10.8 ± 4.8 | 10.7 ± 9.7 | 1.2 ± 1.5 | 6.4 ± 7.1 |
| 6 years | Brij-35 | M | 218 ± 86.5 | 143 ± 18.7 | 113 ± 68.4 | 99.0 ± 12.7 | 36.3 ± 22.2 | 26.5 ± 9.1 | 27.4 ± 27.9 | 0.3 ± 0.6 |
| | | F | 158 ± 51.0 | 93.2 ± 13.5 | 104 ± 31.1 | 45.4 ± 14.0 | 26.1 ± 14.4 | 15.8 ± 11.4 | 12.0 ± 6.3 | 0.7 ± 1.2 |
| | Free | M | 182 ± 62.6 | 116 ± 28.7 | 94.5 ± 49.8 | 83.9 ± 7.4 | 37.1 ± 9.9 | 27.3 ± 2.1 | 28.9 ± 24.1 | 2.6 ± 2.7 |
| | | F | 157 ± 28.3 | 73.2 ± 12.0 | 80.4 ± 28.2 | 37.7 ± 8.4 | 24.6 ± 12.3 | 18.7 ± 10.8 | 20.7 ± 12.4 | 2.7 ± 2.3 |

Values expressed as ng substrate liberated/min./ug DNA ± SD.

M = male; F = female.

Table 22.

Statistical Analysis of Acid Phosphatase Activities Presented in Tables 18 and 19.

Sex Comparisons (M vs. F)

| | | Homogenate | | "Lysosomal" Fraction | | Microsomal Fraction | | Soluble Fraction | |
|---|---|---|---|---|---|---|---|---|---|
| | | WC | SR | WC | SR | WC | SR | WC | SR |
| No Treatment | | | | | | | | | |
| 1 day | Brij-35 | N | N | N | N | N | N | N | S |
| | Free | N | N | N | N | S | N | N | S |
| 6 weeks | Brij-35 | N | S | N | S | N | N | N | N |
| | Free | N | S | N | S | N | N | N | S |
| 6 months | Brij-35 | N | N | N | N | N | N | N | N |
| | Free | N | N | N | N | S | N | N | N |
| 6 years | Brij-35 | N | S | S | N | S | N | N | N |
| | Free | S | N | S | N | N | N | N | N |
| Estrogen Treatment | | | | | | | | | |
| 1 day | Brij-35 | N | S | N | S | N | S | N | S |
| | Free | N | S | N | S | N | S | N | S |
| 6 weeks | Brij-35 | N | S | N | S | N | N | N | N |
| | Free | N | S | N | S | N | N | N | N |
| 6 months | Brij-35 | S | N | S | N | S | N | N | N |
| | Free | S | N | S | N | N | N | N | N |
| 6 years | Brij-35 | N | N | N | S | N | N | N | N |
| | Free | N | N | N | S | N | N | N | N |

S = significantly different ($P < 0.05$); N = not significantly different.

Table 23.

Statistical Analysis of Acid Phosphatase Activities Presented in Tables 18 and 20.·

Age Comparisons

| | | | Homogenate | | "Lysosomal"Fraction | | Microsomal Fraction | | Soluble Fraction | |
|---|---|---|---|---|---|---|---|---|---|---|
| Upper Thoracic | | | WC | SR | WC | SR | WC | SR | WC | SR |
| 1 day - 6 weeks | Brij-35 | M | S | S | N | N | N | S | S | S |
| | | F | N | S | N | S | N | N | N | S |
| | Free | M | S | N | N | S | N | S | N | S |
| | | F | N | S | N | S | N | S | N | N |
| 6 weeks - 6 months | Brij-35 | M | S | N | N | N | N | N | N | N |
| | | F | N | N | N | N | N | N | N | N |
| | Free | M | N | N | N | N | N | N | N | S |
| | | F | N | N | N | N | N | N | N | N |
| 6 months - 6 years | Brij-35 | M | N | N | N | N | N | S | N | N |
| | | F | N | N | N | N | N | N | N | N |
| | Free | M | N | N | N | N | N | N | N | N |
| | | F | N | N | N | N | N | N | N | N |
| Celiac | | | | | | | | | | |
| 6 weeks - 6 months | Brij-35 | M | N | N | N | N | S | N | S | N |
| | | F | N | N | S | N | N | N | N | N |
| | Free | M | N | N | N | N | S | N | N | N |
| | | F | S | N | N | N | N | N | N | N |
| 6 months - 6 years | Brij-35 | M | S | N | N | N | N | N | N | N |
| | | F | S | N | S | N | N | N | S | N |
| | Free | M | S | N | N | N | S | N | N | N |
| | | F | S | N | N | N | N | N | S | N |

S = significantly different ($P < 0.05$); N = not significantly different.
M = male; F = female.

Table 24.

Statistical Analysis of Acid Phosphatase Activities Presented in Tables 19 and 21. Age Comparisons.

| | | | Homogenate | | "Lysosomal" Fraction | | Microsomal Fraction | | Soluble Fraction | |
|---|---|---|---|---|---|---|---|---|---|---|
| Thoracic | | | WC | SR | WC | SR | WC | SR | WC | SR |
| 1 day - | Brij-35 | M | S | S | N | N | S | S | S | S |
| 6 weeks | | F | N | S | N | N | N | S | N | N |
| | Free | M | S | S | S | N | S | S | N | S |
| | | F | N | S | N | N | N | S | N | N |
| 6 weeks - | Brij-35 | M | S | S | N | S | N | S | N | N |
| 6 months | | F | N | N | N | N | S | N | N | N |
| | Free | M | S | S | N | S | N | S | N | N |
| | | F | N | N | N | N | N | N | N | N |
| 6 months - | Brij-35 | M | N | N | N | N | N | N | N | N |
| 6 years | | F | N | N | N | N | S | N | N | N |
| | Free | M | N | N | N | N | N | N | N | N |
| | | F | N | N | N | N | N | N | N | N |
| Celiac | | | | | | | | | | |
| 6 weeks - | Brij-35 | M | N | S | S | S | S | N | N | N |
| 6 months | | F | S | N | S | N | N | N | N | N |
| | Free | M | N | S | S | S | S | N | N | N |
| | | F | S | N | S | N | N | N | N | N |
| 6 months - | Brij-35 | M | S | N | S | S | N | N | S | N |
| 6 years | | F | S | N | S | S | N | N | S | N |
| | Free | M | S | N | S | S | N | N | S | N |
| | | F | S | N | N | S | N | N | S | N |

S = significantly different ($P < 0.05$); N = not significantly different.
M = male; F = female.

Table 25.

Statistical Analysis of Acid Phosphatase Activities Presented in Table 18.

Breed Comparisons (WC vs. SR)

| | | | Homogenate | "Lysosomal" Fraction | Microsomal Fraction | Soluble Fraction |
|---|---|---|---|---|---|---|
| 1 day | Brij-35 | M | S | S | S | S |
| | | F | N | N | N | N |
| | Free | M | S | S | S | S |
| | | F | S | N | N | N |
| 6 weeks | Brij-35 | M | N | N | N | N |
| | | F | N | N | N | N |
| | Free | M | N | N | N | N |
| | | F | N | N | N | N |
| 6 months | Brij-35 | M | N | N | N | N |
| | | F | N | N | N | N |
| | Free | M | S | N | N | N |
| | | F | N | N | N | N |
| 6 years | Brij-35 | M | N | N | N | N |
| | | F | N | N | N | N |
| | Free | M | N | N | N | N |
| | | F | N | N | N | N |

S = significantly different ($P < 0.05$); N = not significantly different.

M = male; F = female.

Table 26.

Statistical Analysis of Acid Phosphatase Activities Presented in Table 19.

Breed Comparisons (WC vs. SR)

| | | | Homogenate | "Lysosomal" Fraction | Microsomal Fraction | Soluble Fraction |
|---|---|---|---|---|---|---|
| 1 day | Brij-35 | M | S | S | S | S |
| | | F | N | N | S | N |
| | Free | M | S | S | N | S |
| | | F | N | N | S | N |
| 6 weeks | Brij-35 | M | S | N | N | N |
| | | F | N | N | N | N |
| | Free | M | S | N | N | N |
| | | F | N | N | N | N |
| 6 months | Brij-35 | M | S | S | N | N |
| | | F | N | N | N | N |
| | Free | M | S | S | N | N |
| | | F | N | N | N | N |
| 6 years | Brij-35 | M | N | N | N | N |
| | | F | N | N | N | N |
| | Free | M | N | N | N | N |
| | | F | N | N | N | N |

S = significantly different ($P < 0.05$); N = not significantly different.

M = male; F = female.

Table 27.

Statistical Analysis of Acid Phosphatase Activities Presented in Tables 20 and 21.

Sex Comparisons (M vs. F )

| Celiac Region | | Homogenate | | "Lysosomal" Fraction | | Microsomal Fraction | | Soluble Fraction | |
|---|---|---|---|---|---|---|---|---|---|
| | | WC | SR | WC | SR | WC | SR | WC | SR |
| No treatment | | | | | | | | | |
| 6 weeks | Brij-35 | N | N | N | S | N | N | N | N |
| | Free | N | N | S | S | N | N | N | N |
| 6 months | Brij-35 | S | N | S | N | S | N | N | N |
| | Free | S | S | N | S | S | N | N | N |
| 6 years | Brij-35 | S | N | N | S | S | N | S | N |
| | Free | N | S | N | S | S | N | S | N |
| Estrogen Treatment | | | | | | | | | |
| 6 weeks | Brij-35 | N | S | N | S | N | N | N | N |
| | Free | N | S | N | S | N | N | N | N |
| 6 months | Brij-35 | S | N | N | N | S | S | N | N |
| | Free | S | N | N | N | S | S | N | N |
| 6 years | Brij-35 | S | S | N | S | N | N | N | N |
| | Free | N | S | N | S | N | N | N | N |

S = significantly different ($P < 0.05$); N = not significantly different.

Table 28.

Statistical Analysis of Acid Phosphatase Activities Presented in Table 20.

Breed Comparisons (WC vs. SR)

| | | | Homogenate | "Lysosomal"Fraction | Microsomal Fraction | Soluble Fraction |
|---|---|---|---|---|---|---|
| 6 weeks | Brij-35 | M | N | S | S | S |
| | | F | N | S | N | N |
| | Free | M | S | S | N | N |
| | | F | S | N | N | N |
| 6 months | Brij-35 | M | N | N | S | N |
| | | F | N | N | N | N |
| | Free | M | N | N | N | N |
| | | F | N | N | N | N |
| 6 years | Brij-35 | M | S | N | S | N |
| | | F | S | N | N | S |
| | Free | M | S | N | S | N |
| | | F | S | N | N | S |

S = significantly different ($P < 0.05$); N = not significantly different.

M = male; F = female.

Table 29.

Statistical Analysis of Acid Phosphatase Activities Presented in Tables 18 and 20.

Aortic Site Comparisons (Thoracic vs. Celiac)

| | | | Homogenate | | "Lysosomal" Fraction | | Microsomal Fraction | | Soluble Fraction | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | WC | SR | WC | SR | WC | SR | WC | SR |
| 6 weeks | Brij-35 | M | S | N | N | N | N | S | S | N |
| | | F | N | N | N | N | N | N | N | N |
| | Free | M | S | N | N | N | N | N | N | N |
| | | F | N | N | N | N | N | N | N | N |
| 6 months | Brij-35 | M | N | N | N | N | S | S | N | N |
| | | F | N | N | N | N | N | N | N | N |
| | Free | M | N | N | N | N | N | S | N | N |
| | | F | N | N | N | N | N | N | N | N |
| 6 years | Brij-35 | M | S | N | S | N | S | N | N | N |
| | | F | S | N | S | N | N | N | S | N |
| | Free | M | S | N | N | N | S | N | N | N |
| | | F | S | N | S | N | N | N | S | N |

S = significantly different ($P < 0.05$); N = not significantly different.

M = male; F = female.

Table 30.

Statistical Analysis of Acid Phosphatase Activities Presented in Table 21

Breed Comparisons (WC vs. SR)

| | | | Homogenate | "Lysosomal" Fraction | Microsomal Fraction | Soluble Fraction |
|---|---|---|---|---|---|---|
| 6 weeks | Brij-35 | M | N | N | N | N |
| | | F | N | S | N | N |
| | Free | M | N | N | N | N |
| | | F | S | S | S | N |
| 6 months | Brij-35 | M | N | N | N | N |
| | | F | N | S | N | N |
| | Free | M | N | N | S | N |
| | | F | N | N | N | N |
| 6 years | Brij-35 | M | S | N | N | S |
| | | F | S | S | N | S |
| | Free | M | S | N | N | S |
| | | F | S | S | N | S |

S = significantly different ($P < 0.05$); N = not significantly different.

M = male; F = female.

Table 31.

Statistical Analysis of Acid Phosphatase Activities Presented in Tables 19 and 20. Aortic Site Comparisons (Thoracic vs. Celiac)

| | | | Homogenate | | "Lysosomal" Fraction | | Microsomal Fraction | | Soluble Fraction | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | WC | SR | WC | SR | WC | SR | WC | SR |
| 6 weeks | Brij-35 | M | S | S | S | N | N | S | N | N |
| | | F | N | N | S | N | N | N | N | N |
| | Free | M | S | S | S | N | N | S | N | N |
| | | F | N | N | N | N | N | N | N | N |
| 6 months | Brij-35 | M | N | N | N | N | S | S | N | N |
| | | F | N | N | N | N | N | N | N | N |
| | Free | M | N | N | N | N | S | S | N | N |
| | | F | N | N | N | N | N | N | N | N |
| 6 years | Brij-35 | M | S | N | N | N | S | N | S | N |
| | | F | N | N | N | N | N | N | N | N |
| | Free | M | S | N | N | N | S | N | S | N |
| | | F | S | N | S | N | N | N | S | N |

S = significantly different ($P < 0.05$); N = not significantly different.

M = male; F = female.

Table 32.

Percentage of Latent Acid Phosphatase Activities in the "Lysosomal" Fraction

| | | Upper Thoracic | | | | Celiac Bifurcation | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | WC | | SR | | WC | | SR | |
| | | Control | Estrogen | Control | Estrogen | Control | Estrogen | Control | Estrogen |
| 1 day | | | | | | | | | |
| M | Actual | 6.4 ± 0.1 | 18 ± 10 | 13 ± 7 | 17 ± 3 | | | | |
| | % | 12 ± 1 | 29 ± 12 | 13 ± 6 | 15 ± 8 | | | | |
| F | Actual | 9.1 ± 8.5 | 12 ± 4 | 5.8 ± 1 | 11 ± 4 | | | | |
| | % | 18 ± 16 | 19 ± 4 | 8 ± 1 | 19 ± 4 | | | | |
| 6 weeks | | | | | | | | | |
| M | Actual | 4.5 ± 4.5* | 3.5 ± 3.5* | 13 ± 11* | 21 ± 5 | 2 ± 2* | 23 ± 4 | 19 ± 7 | 28 ± 17 |
| | % | 6 ± 8 | 5 ± 5 | 17 ± 13 | 21 ± 1 | 2 ± 2 | 19 ± 4 | 24 ± 7 | 21 ± 6 |
| F | Actual | 23 ± 9 | 11 ± 17* | 8.2 ± 2.4 | 3.3 ± 2.4* | 30 ± 8 | 31 ± 13 | 6.7 ± 5.0* | 15 ± 4 |
| | % | 30 ± 5 | 16 ± 13 | 17 ± 3 | 7 ± 4 | 35 ± 10 | 27 ± 12 | 16 ± 10 | 25 ± 6 |
| 6 months | | | | | | | | | |
| M | Actual | 14 ± 10* | 20 ± 10 | 14 ± 5 | 16 ± 10 | 24 ± 14 | 25 ± 11 | 15 ± 20* | 26 ± 7 |
| | % | 16 ± 11 | 21 ± 9 | 18 ± 4 | 24 ± 13 | 25 ± 13 | 35 ± 15 | 16 ± 20 | 28 ± 2 |
| F | Actual | 6.2 ± 12* | 5.1 ± 10* | 18 ± 7.0 | 12 ± 8 | 4.3 ± 2.9* | 3.3 ± 5.4* | 17 ± 18* | 19 ± 1 |
| | % | 10 ± 10 | 10 ± 10 | 32 ± 12 | 21 ± 16 | 9.5 ± 8.4 | 5.8 ± 6.6 | 23 ± 22 | 29 ± 1 |
| 6 years | | | | | | | | | |
| M | Actual | 17 ± 11* | 20 ± 12* | 6.2 ± 8* | 15 ± 8 | 27 ± 4 | 38 ± 19 | 18 ± 8 | 15 ± 12* |
| | % | 21 ± 12 | 21 ± 12 | 8 ± 10 | 18 ± 9 | 24 ± 6 | 29 ± 3 | 18 ± 8 | 15 ± 11 |
| F | Actual | 12 ± 5 | 19 ± 8 | 11 ± 5* | 9 ± 2 | 24 ± 8 | 24 ± 4 | 9.4 ± 19.1* | 7.7 ± 6.6* |
| | % | 23 ± 7 | 28 ± 13 | 18 ± 10 | 16 ± 3 | 26 ± 6 | 23 ± 6 | 20 ± 19 | 17 ± 13 |

Actual = actual difference between Brij-35 activity and Free activity in ng/min./ug DNA.
% = % Latency
* Not significantly different from o ($P < 0.05$)
M = male; F = female.

Table 33.

Statistical Analysis of Latent Acid Phosphatase Activities Presented in Table 32.

Breed (WC vs. SR), Aortic Site (Thoracic vs. Celiac), and Treatment (Control vs. Estrogen) Comparisons

| | | | Breed Comparisons | | | | Aortic Site Comparisons | | | | Treatment Comparisons | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Upper Thoracic | | Celiac Bifurcation | | WC | | SR | | Upper Thoracic | | Celiac | |
| | | | Control | Estrogen | Control | Estrogen | Control | Estrogen | Control | Estrogen | WC | SR | WC | SR |
| 1 day | M | Actual | N | N | | | | | | | S | N | | |
| | | % | N | N | | | | | | | N | N | | |
| | F | Actual | N | N | | | | | | | N | N | | |
| | | % | N | N | | | | | | | N | S | | |
| 6 weeks | M | Actual | N | N | S | N | N | S | N | N | N | N | S | N |
| | | % | N | N | S | N | N | N | N | N | N | N | S | N |
| | F | Actual | S | N | S | N | N | N | N | S | N | N | N | N |
| | | % | S | N | N | N | N | N | N | S | N | S | N | N |
| 6 months | M | Actual | N | N | N | N | N | N | N | N | N | N | N | N |
| | | % | N | N | N | N | N | N | N | N | N | N | N | N |
| | F | Actual | N | N | N | S | N | N | N | N | N | N | N | N |
| | | % | S | S | N | S | N | N | N | N | N | N | N | N |
| 6 years | M | Actual | N | N | N | N | N | N | N | N | N | N | N | N |
| | | % | N | N | N | N | N | N | N | N | N | N | N | N |
| | F | Actual | N | N | N | S | N | N | N | N | N | N | N | N |
| | | % | N | N | N | N | N | N | N | N | N | N | N | N |

S = significantly different ($P < 0.05$); N = not significantly different.

M = male; F = female.

Table 34.

Statistical Analysis of Latent Acid Phosphatase Activities Presented in Table 32.
Sex Comparisons (M vs. F).

| | | Upper Thoracic Aorta | | | | Celiac Bifurcation | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | WC | | SR | | WC | | SR | |
| | | Control | Estrogen | Control | Estrogen | Control | Estrogen | Control | Estrogen |
| 1 day | Actual | N | N | N | N | | | | |
| | % | N | N | N | N | | | | |
| 6 weeks | Actual | S | N | N | S | S | N | N | N |
| | % | S | N | N | S | S | N | N | N |
| 6 months | Actual | N | N | N | N | S | S | N | N |
| | % | N | N | S | N | N | S | N | N |
| 6 years | Actual | N | N | N | N | N | N | N | N |
| | % | N | N | N | N | N | N | N | N |

S = significantly different ($P < 0.05$); N = not significantly different.

BIOGRAPHICAL DATA

| | |
|---|---|
| Name in Full | Jane Linscott Hough |
| Date of Birth | March 29, 1950 |
| Place of Birth | Morristown, New Jersey |
| Secondary Education | Kent Place School<br>Summit, New Jersey |

| Collegiate Institutions attended | Dates | Degrees |
|---|---|---|
| Middlebury College | 1968-1972 | B.A. |
| University of New Hampshire | 1975-1980<br>awarded 1981 | Ph.D |

Honors or Awards

National Science Foundation Summer Fellowship

University of New Hampshire Teaching Assistants
Summer Fellowship

New Hampshire Heart Association Pre-doctoral Fellowship

Sigma Xi

Publications none

| Positions Held | Dates |
|---|---|
| Technician<br>Div. of Nutritional Sciences<br>Cornell University<br>Cornell, New York | 1980-1981 |
| Research Assistant<br>Dept. of Animal Sciences<br>University of New Hampshire<br>Durham. New Hampshire | 1978-1980 |
| Teaching Assistant<br>Dept. of Biochemistry<br>University of New Hampshire<br>Durham, New Hampshire | 1975-1977 |

| Positions Held | Dates |
| --- | --- |
| Technician<br>Marine Colloids<br>Springfield, New Jersey | 1974-1975 |
| Technician<br>The Research Corporation of New England<br>Whethersfield, Connecticut | 1973-1974 |
| Summer Internship<br>Bell Telephone Laboratories<br>Murray Hill, New Jersey | 1972 |