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DAVID PHILLIP HAJJAR

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BIOENERGETIC METABOLISM AND LIPID ACCUMULATION IN PIGEON ATHEROGENESIS.

UNIVERSITY OF NEW HAMPSHIRE, PH.D., 1978
BIOENERGETIC METABOLISM AND LIPID ACCUMULATION
IN PIGEON ATHEROGENESIS

by

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B.A. American International College, 1974
M.S. University of New Hampshire, 1977

A DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy
in
Biochemistry

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ACKNOWLEDGEMENTS

I express my sincere gratitude to Dr. Samuel Smith who has been completely selfless in offering his attention to my work and training at this university. His high academic and scientific standards have been a constant source of inspiration.

Appreciation is extended to the members of my doctoral committee for their participation and contributions to the broadening of my training program and the preparation of this thesis.

The author also expresses his gratitude to Dr. W. C. Skoglund for the opportunity to conduct this work in the Department of Animal Sciences. Furthermore, I thank Dr. Edward Tillinghast for the privilege of using his research facilities so that I could conduct the oxidative phosphorylation experiments.

Special acknowledgement must also go to Helen Langley, Shirley Robie, Emory Clippert, and Angela Stucchi for their technical assistance.

With fond memories, I thank some of my fellow graduate students who often constituted a willing forum for scientific discussion.

Also, I am grateful to my family for their unending support and encouragement during the two decades I have been in school.

Finally, the financial support provided in the form of New Hampshire Heart Association Pre-doctoral Fellowship is gratefully acknowledged.
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ABSTRACT

BIOENERGETIC METABOLISM AND LIPID ACCUMULATION IN PIGEON ATHEROGENESIS

by

DAVID PHILLIP HAJJAR

Alterations in energy metabolism, particularly in coupled oxidative phosphorylation and pyridine nucleotide-linked (ATP) transhydrogenation, during early spontaneous atherogenesis may be related to lipid accumulation in lesion progression as a cause, result, or concurrent effect. The purpose of this investigation was to determine whether lipid accretion, the key pathological feature of atherogenesis, occurs before, after, or at the same time as alterations in energy metabolism in atherosclerosis-susceptible White Carneau (WC) pigeon aortas. Spontaneous atherosclerotic lesions in the WC closely resemble those in human aortas, and they are highly predictable in site specificity and rate of pathogenic progression. Aortas from atherosclerosis-resistant Show Racer (SR) pigeons were used as controls to permit discrimination between changes due to normal aging and those due to pathological involvement in WC.
Lipid from thoracic aortas and celiac cushions, a site predisposed to lesion formation, of WC pigeons was isolated, separated chromatographically into six classes, quantitated fluorometrically, and compared with that from corresponding SR aortic sites at hatching and throughout a period corresponding to initial biochemical and ultrastructural abnormalities (six weeks, twelve weeks, and six months of age).

Thoracic aortas from both breeds contained similar amounts and proportions of cholesterol, free fatty acids, triacylglycerols, cholesteryl esters, squalene, and phospholipids at corresponding ages, and showed nearly identical aging profiles for individual lipid classes and total lipids. However, six-month old WC thoracic aortas had more saturated free fatty acids and less unsaturated fatty acids than SR aortas. Thoracic aortas contained significantly less total lipid than the celiac cushions at six weeks and subsequent ages in WC and at twelve weeks and subsequent ages in SR.

Celiac sites in WC contained significantly greater amounts of total lipid than SR celiac foci at six weeks and six months of age. The difference at six weeks of age was due primarily to free fatty acids and cholesterol; however, there were significant breed differences in all lipid classes except squalene by six months of development. At this age, phospholipids and squalene comprised the largest proportion of lipid in celiac sites in both breeds. Six-month WC celiac cushions also contained more saturated and less unsaturated fatty acids than SR cushions.

Lack of control of NADP+ reduction via NADH by ATP in WC celiac foci at six weeks and subsequent ages was observed. Since this process is involved in control of fatty acid synthesis by regulating the NADPH/NADP+ ratio.
ratio, this may explain high concentrations of free fatty acids in WC celiac foci at six weeks and subsequent ages.

Measurements of oxidative phosphorylation demonstrated less coupled respiratory-chain phosphorylation in six-month old WC celiac foci when compared to corresponding SR aortic sites with either $\beta$-hydroxybutyrate or succinate as substrate. This was due primarily to the inability of WC celiac mitochondria to utilize inorganic phosphate at rates similar to the SR.

This study has suggested a relationship between the lack of control of NADH transhydrogenation and early lipid accretion in WC celiac cushions. Initial lipid accumulation, potentiated by this lack of control of NADH transhydrogenation, occurs prior to uncoupled oxidative phosphorylation. The process of lipid accretion may be a contributing factor to altered oxidative metabolism which can in turn further exacerbate biochemical arteriopathy in the WC. Unlike the metabolic situation described in the WC, it is suggested that tighter coupling of oxidative phosphorylation in six-month old SR aortas may enhance the ability of this breed to withstand stresses associated with atherogenesis at the celiac focus.
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INTRODUCTION

Cardiovascular diseases have been directly implicated in approximately one million deaths annually in this country alone. Specifically, atherosclerosis is considered the primary killer in the United States and much of the industrialized world (Ross and Glomset, 1973). This prompted the World Health Organization's 1969 proclamation that cardiovascular disease was becoming the most widespread epidemic ever to affect mankind. In spite of seventy-five years of research, atherosclerosis remains a major world health problem. Consequently, study of the etiology of this disease has been a major priority for medical researchers in this country and abroad.

The multifactorial pathogenesis of atherosclerosis is now well recognized (Davignon, 1978). Since the atherosclerotic lesion may be reversible and/or preventable (Dalferes et al., 1971), it is important to understand the pathogenesis and to identify initiating factors. In the complex chain of events which leads to development of the atheromatous plaque, three levels of influence may be considered. First, the genetic propensity provides a favorable setting within the arterial wall. At a second level of influence, these arterial wall factors interact with components of the circulating blood to promote formation of the plaque. Constituents of the arterial wall may contribute to the process. Finally, dietary factors, smoking, hypertension, hyperlipidemia, and diabetes are but a few of the systemic risk factors presumably influencing interactions between the blood and the arterial wall. Their effect on the arterial wall may be mediated by such factors.
as low or high density lipoproteins, hemodynamic stress, lowered oxygen
tension (hypoxia), insulin or other hormone levels, or blood cell
properties and constituents. Moreover, the response of the artery
will depend on the permeability of the endothelium, the structure of
the arterial wall, and its metabolic activity.

Focal differences in the metabolism of the arterial wall, perhaps
genetically controlled or in response to hemodynamic stress, may be
important during atherogenesis. Specifically, there has been consider­
able research during the past two decades aimed at elucidating the role
of vascular smooth muscle cell metabolism in the development of athero­
sclerotic lesions (Hajjar and Smith, 1978; Kalra and Brodie, 1974;
Morrison et al., 1974; Whereat, 1967; Zemplenyi, 1968; Zemplenyi et al.,
1975). For instance, when aerobic energy metabolism is considered,
variations in mitochondrial morphology (Watts, 1963) and in oxidative
metabolism of aortic smooth muscle cells (Santerre et al., 1974; Scott
et al., 1969) have been found to occur prior to the development of
atherosclerotic plaques. Plaque development is accompanied by a
stimulation of several specific metabolic processes which require
energy (via anaerobic and aerobic mechanisms) such as DNA synthesis
and cell division (Thomas et al., 1968), protein synthesis (Kim et al.,
1968), and fatty acid and complex-lipid synthesis (Lofland et al., 1965;
St. Clair et al., 1968a; 1969).

Lipid accretion also plays a key role in the pathogenesis of lesion
formation during spontaneous atherogenesis, particularly at branched
portions of thoracic aortas (Clarkson et al., 1959; Nicolosi et al.,
1972). Many review articles have been written summarizing the relation­
ship of various tissue lipids and their influence on the development of
atherosclerosis (Portman, 1970; Small, 1977; Smith, 1974; St. Clair, 1976). Such atherogenic lipid accumulation has been related to hemodynamic irregularities (Caro et al., 1969; Haimovici, 1968; Texon, 1974), accumulation of glycosaminoglycans (Curwen and Smith, 1977), altered energy metabolism (Hajjar and Smith, 1978; Kalra and Brodie, 1974; Santerre et al., 1974), and focal hypoxia (Farber et al., 1978).

The avascularity of aortic medial tissue and its dependence on diffusion mechanisms for oxygen supply makes it susceptible to hypoxia (Kirk, 1963; Whereat, 1967). It has been further suggested by Simard-Duquesne and Allard (1967) that diffusion barriers (such as lipid) may exist which could limit oxygen availability to the middle aortic wall. If this situation becomes aggravated, eventual impairment of mitochondrial function can occur, curtailing energy production by causing uncoupling of oxidative phosphorylation (Whereat, 1967; Zemplenyi, 1968). Low oxygen tension has been shown to affect energy dynamics by causing decreases in phosphorylation rates, decreased levels of oxidative enzymes, and increased glycolysis (Bartley et al., 1968).

At aortic sites predisposed to lesion formation, focal hypoxia and ischemia, aggravated by pre-existing lipid or other blood-derived products in the vascular wall (Mergner et al., 1977), have also been shown to stimulate lipid synthesis (Filipovic and Buddecke, 1971; Gordon et al., 1977; Kinnula and Hassinen, 1978). Low oxygen availability can not only stimulate greater lipoidosis (Myasnikov, 1958) but also cause a decrease in the cyclization process of squalene to cholesterol, allowing squalene to accumulate (Lazzarini-Robertson, 1968). Since mammalian cells are not able to utilize squalene in large amounts, the increased accumulation of squalene coupled with increased perme-
ability of the smooth muscle cell membrane to extracellular lipid during hypoxia may accelerate formation of foam cells (Lazzarini-Robertson, 1968). Consequently, squalene accumulation has been correlated with the severity of atherosclerosis (St. Clair et al., 1968a). In similar studies, Lazzarini-Robertson (1968) demonstrated increased incorporation of extracellular lipids in vitro into human intimal cells cultured under low oxygen tension. If similar permeability responses occur in vivo, accumulating lipid could subsequently impair oxidative phosphorylation in the mitochondria of these cells (Vasquez-Colon et al., 1966).

By interfering with the arterial wall, hypoxic conditions can not only stimulate incorporation and synthesis of certain lipids, but it can also inhibit the synthesis of phospholipids needed for oxidative metabolism in aortic muscle cells (Lazzarini-Robertson, 1968; Racker, 1970). If there is excess intracellular cholesterol, the emulsifying capacity of pre-existing phospholipids becomes reduced. Furthermore, if increased oxygen supply and energy (ATP) demands are not satisfied, lipid micelles begin to be transformed into 'globular fat' which cannot be easily mobilized by the cell thus accelerating the atherosclerotic pathology (Lazzarini-Robertson, 1968).

Several studies have directly implicated mitochondrial involvement with lipid accumulation in the pathogenesis of atherosclerosis, viz., in the loss of ATPase activity (Watts, 1963). Histochemical studies by Smith et al. (1966) demonstrated lower succinate dehydrogenase and mitochondrial ATPase activity in essential fatty acid-deficient mitochondria of cultured aortic intimal cells from atherosclerosis-susceptible White Carneau (WC) pigeons when compared with atherosclerosis-resistant Show Racer (SR) cells. Uncoupled oxidative
phosphorylation was proposed as a contributing factor to lipid vacuole formation which occurred in these cultured WC cells. Hajjar and Smith (1978) further proposed a decrease in coupled oxidative phosphorylation during spontaneous atherogenesis based on lower activity of coupled mitochondrial $F_1$ ATPase in six-month old WC pigeon celiac foci compared to SR celiac foci. This decline in energy transducing capability of WC aortas may reflect decreased ability of this organ to eliminate intracellular materials.

Other studies have related lipid metabolism to aberrations of energy metabolism during various stages of spontaneous atherosclerosis. Kalra and Brodie (1974) demonstrated an increase in lactic acid and glycerol-3-phosphate accumulation in twelve-week old WC aortic tissue as compared to SR of the same age. The observed deficiency of the glycerol phosphate shuttle, as shown by a decrease in glycerol phosphate dehydrogenase activity, could promote lipogenesis by increasing glycerol phosphate available for conversion to phospholipids and triacylglycerols. The increased steady state levels of lactate and glycerol phosphate and the decrease in lipoamide dehydrogenase activity in WC aortic smooth muscle cells (Zemplenyi and Rosenstein, 1975) indicate that aging WC aorta cells may obtain their energy by anaerobic means. Since arterial smooth muscle cells can rely on anaerobic metabolism such as glycolysis even in the presence of oxygen (Lehninger, 1959; Zemplenyi, 1968), it appears that the atherosclerotic arterial wall has a deficient Pasteur effect (Lehninger, 1959). This can result in inefficient utilization of glucose by the arterial wall since only five percent as much ATP is produced by glycolysis compared with that provided by complete oxidation of glucose via the tricarboxylic acid (TCA) cycle.
(Lazzarini-Robertson, 1968). Consequently, a weak Pasteur effect suggests a defect in coupled respiration which could lead to decreasing levels of ATP, especially under conditions of metabolic stress or lipid overloads (Lehninger, 1959).

Since the original work by Kirk and co-workers (1954) on respiration and glycolysis of arterial tissue, a number of investigations have reported changes in glucose metabolism and oxygen consumption in atherosclerotic tissue. For reviews, see Kirk (1969) and Zemplenyi (1968). These earlier studies showed that arterial tissue utilizes considerable amounts of glucose, producing large amounts of lactic acid. More recently, St. Clair et al. (1974) demonstrated a two-fold increase in glucose utilization with virtually all of this excess glucose being metabolized to lactate in the atherosclerotic blood vessel wall.

Oxygen consumption increased about two-fold in the atherosclerotic vessel along with lipid accumulation, yet there was little pyruvate entering the Krebs cycle to account for this increase in oxygen consumption (Morrison et al., 1972). Consequently, the increased oxygen consumption of the atherosclerotic artery is not primarily due to increased glucose metabolism via the Krebs cycle, but may be attributed to \( \beta \)-oxidation of fatty acids (Morrison et al., 1974) or oxygen utilizing enzymes such as mixed function oxygenases (St. Clair, 1976).

These studies describing alterations in energy metabolism closely parallel the recent work by Zemplenyi et al. (1975) who demonstrated a shift in energy production from the tricarboxylic acid cycle (TCA) to anaerobic glycolysis in five-to eight-week old WC aortas. Less TCA cycle activity in WC aortas compared to SR aortas could retard fatty acid oxidation and favor synthesis of lipids during early atherogenesis.
by making more citrate (or acetate) available in the cytoplasm. Furthermore, with decreased oxidative catabolism as demonstrated by lower TCA cycle activity, less ATP is produced per glucose molecule oxidized (Lehninger, 1975). This indicates less efficient energy production which can eventually produce diminished catabolism. It has been speculated that 'sluggish' metabolism may be responsible for the diminished efflux of lipids from fatty streaks and plaques developing along the arterial wall (Adams, 1973; Newman and Zilversmit, 1966). The presence of large amounts of lipid, particularly cholesteryl esters at aortic lesion sites has been demonstrated in aortas from several animal species including humans by several investigators who were able to correlate cholesteryl ester accumulation with the severity of atherosclerosis (Day and Wahlquist, 1970; Dayton and Hashimoto, 1968; Lofland et al., 1965; Smith, 1965).

In other studies, Kalra and Brodie (1974) demonstrated lack of control of NADP⁺ reduction by the energy-linked (ATP) NADH transhydrogenase in mitochondria from three-to four-month old WC thoracic aortas. Since the NADPH/NADP⁺ ratio in cells can control the synthesis of triacylglycerols and fatty acids (Kalra and Brodie, 1973), it is likely that an increase in the NADPH/NADP⁺ ratio influences accumulation of fatty acids and triacylglycerols in diseased arteries (Whereat, 1970). Moreover, Wojtczak (1976) has reported that accumulation of free fatty acids, particularly palmitic, stearic, and oleic acids, can affect energy metabolism since they are potent uncouplers of respiratory-chain phosphorylation. The arterial wall obtains approximately 50% of its ATP via oxidative phosphorylation (Scott et al., 1970). If the energy dynamics of the blood vessel wall are altered by accumulating fatty
acids, this could seriously affect intermediary metabolism during the disease process (Whereat, 1967).

Preliminary studies have shown that lipid accumulation and uncoupled oxidative phosphorylation are both characteristics of early "spontaneous" atherogenesis at aortic lesion sites (Nicolosi et al., 1972; Santerre et al., 1974). On the basis of state 3 and state 4 respiration rates (oxygen consumption in the presence and absence of ADP, respectively) and respiratory (acceptor) control ratios, Santerre et al. (1974) suggested a defect in the systems coupling oxidative phosphorylation to electron transport in pre-atherosclerotic celiac foci of six-month old WC pigeons. However, analysis of the lipid content and composition of these foci by Nicolosi et al. (1972) showed that excessive amounts of squalene and cholesterol esters were already present in the pre-atherosclerotic celiac site. Therefore, it may be possible that lipid in the pre-atherosclerotic celiac site is responsible for uncoupled respiratory-chain phosphorylation rather than having accumulated as a result of defective mitochondrial metabolism (Klingenberg and Bode, 1965). It is evident that the sequence of these pathological events must be elucidated prior to searching for causitive factors during early spontaneous atherogenesis since: (1) lipid accumulation may result from a defect in oxidative mitochondrial metabolism, particularly in coupled oxidative phosphorylation (Klingenberg and Bode, 1965; Spector, 1971; Whereat, 1967); (2) lipid accumulation can cause a loss of oxidative energy production (Klingenberg and Bode, 1965; Santerre et al., 1974; Vasquez-Colon et al., 1966; Wojtczak, 1976); or (3) altered energy metabolism and lipid accumulation may occur simultaneously as a result of some previous abnormality.
However, it should be emphasized that studies which provide evidence supporting alterations in either lipid metabolism or in the bioenergetics of the arterial wall as the earlier event during spontaneous atherogenesis are difficult to interpret because: (1) large areas of uninvolved aortic tissue have been included in many lipid analyses and in the study of oxidative energy metabolism thereby diluting or masking changes in focally affected regions; (2) many studies of the bioenergetics of the arterial wall report only oxygen consumption rates and not P/O ratios with acceptor control ratios; (3) many results derived from experimentally-induced atheroarteriosclerosis often cannot be easily extrapolated to the spontaneous disease process; and (4) studies on lipid metabolism are often based on dry or wet weights which are subject to variations due to inert extracellular material in aortic tissue.

Objectives of the Study

Since it still remains unclear whether changes in oxidative energy production in vascular smooth muscle cell metabolism at aortic lesion sites may be caused by lipid accretion or vice versa, the aim of this study was to assemble a sequential pattern of metabolic events describing early spontaneous atherogenesis in the WC by:

(1) identifying the initial period of spontaneous lipid accumulation in the celiac site;

(2) characterizing the type(s) of lipid accumulating during early spontaneous atherogenesis particularly at aortic sites predisposed to lesion formation;

(3) profiling as a function of age and aortic site the energy-linked
(ATP) NADH transhydrogenase reaction, i.e.,

$$\text{NADH} + \text{NADP}^+ + \text{energy (ATP)} \rightarrow \text{NAD}^+ + \text{NADPH}$$

which can produce NADPH for the biosynthesis of lipid; and,

(4) assessing the capacity of WC and SR vascular smooth muscle cells
for coupled oxidative phosphorylation as a function of age and
aortic site; thereby, determining the sequence of appearance of
aberrations of energy metabolism and initial lipid accretion.

Coupled respiratory-chain phosphorylation was evaluated by oxygen
polarigraphy as indicated by P/O ratios. These ratios are presented
in order to further investigate the findings of Santerre et al. (1974)
who proposed a loss of integrity of oxidative phosphorylation in four-
to six-month old WC celiac cushions based on low respiratory control
ratios (RCRs). Both parameters (P/O and R C ratios) are used to
describe changes in the control of coupling and phosphorylation (Kalra
and Brodie, 1973; 1974; Mergner et al., 1977; Simard-Duquesne, 1969).
However, it should be emphasized that they do not measure the same
events and cannot be used interchangeably (Lehninger, 1964). If the
mitochondria are loosely coupled, a direct measure of phosphate
utilization must also be examined. The respiratory (or acceptor)
control ratio (RCR) is based on different rates of oxygen consumption
supported by substrate (electron donor) with (state 3) and without
(state 4) a phosphate acceptor (ADP). Acceptor control ratios measure
the "efficiency" of ADP conversion to ATP (Morrison and Scott, 1974).
The P/O ratio determines the utilization of inorganic phosphate per
oxygen atom consumed and, therefore, the production of ATP by the mito-
chondrion. It is an index of how tightly coupled oxidative phosphorylation is to electron transport in the mitochondria (Trump and Arstila, 1975).

**Animal Models as Experimental Tools**

In order to test the hypothesis of altered metabolic capabilities in the muscular foci at the celiac artery bifurcation, an animal system was needed which contained individuals differing in susceptibility to spontaneous atherosclerosis. Atherosclerosis-susceptible White Carneau (WC) pigeons and atherosclerosis-resistant Show Racer (SR) pigeons (control) provide a unique system for the study of metabolic variations in aortic smooth muscle cells since the pathogenesis of atherosclerosis in WC pigeons is similar to the human condition, and the atheromatous lesions are highly predictable in terms of site specificity at the celiac bifurcation and in the rate of pathogenic progression as a function of age (Clarkson et al., 1973; Prichard et al., 1962; Santerre et al., 1972; St. Clair et al., 1968a).
MATERIALS AND METHODS

1. Derivation of Experimental Tissue

One-to-three-day old pigeons were obtained from our colonies which were originally established with birds from Palmetto Pigeon Plant (Sumter, South Carolina) of the same lines as described by Clarkson et al. (1959). Six-week old, twelve-week old, and six-month old WC and SR pigeons were obtained from Palmetto Pigeon Plant. The birds were housed in fly coops with free access to water, Purina Pigeon Pellets, and Palmetto Health Grit.

2. Total Lipid and DNA Analyses

A. General Procedure

Pigeons were sacrificed by exsanguination after which the sex of each pigeon was determined by dissection. The upper thoracic aorta and the celiac bifurcation were excised immediately and placed in chilled (4°C) Hanks Balanced Salt Solution without glucose (HBSS(-)). Adherent blood, and perivascular and adventitial tissue were carefully stripped and dissected away after the celiac cushions were separated by dissection from the upper thoracic aortas. Tissue for lipid analyses were pooled and stored frozen at -70°C in air-tight vials (4.5 mm X 1.0 mm) under N₂(g) for no longer than eight weeks. Aortic tissue used for free fatty acid profile analyses was fresh and not stored. Four pools of aortic tissue for each age-site combination from each breed were analyzed for lipid. Each pool contained segments of aortic tissue from birds of both sexes to provide at least 10 mg
wet tissue weight.

Following homogenization of aortic tissue in 1.0 ml physiological saline (pH 7.0) with a manual, glass microhomogenizer, a 0.3 ml aliquot was taken for DNA analysis by the micro-fluorometric method of Kissane and Robins (1958) using 3,5-diaminobenzoic acid. Tissue aliquots for DNA determination were treated with 0.9% Pronase (Calbiochem, nuclease-free) in 0.20 M Tris-HCl, pH 8.0, at 37°C for 12 hours to release all cellular DNA prior to analysis (Figure 1, page 29). Lipids were extracted from the remaining 0.70 ml aliquot by the method of Folch et al. (1957). (All glassware for lipid analyses was previously cleaned in dichromate-sulfuric acid and rinsed thoroughly with distilled water.) Since the lipids were to be quantitated fluorometrically, the lipid extract was hydrogenated for 5 hours with platinum oxide as a catalyst (Farquhar et al., 1959) in order to prevent quenching of the fluorescent spots due to the presence of double bonds. (GLC analysis confirmed that the hydrogenation process removed all unsaturated fatty acids.) This lipid extract was then filtered through a sintered-glass filter to remove the catalyst. The solution was evaporated to dryness under N₂(g), and the residue was dissolved in dichloromethane: methanol (2:1, v/v). (All organic solvents were reagent grade and distilled prior to use.) The final extract volume was prepared to 10.0 ml. A 3.0 ml aliquot was subsequently fractionated by thin-layer chromatography into six lipid classes: phospholipids, cholesterol, non-esterified fatty acids, triacylglycerols, cholesteryl esters, and squalene using a two solvent system (Hojnacki and Smith, 1974). All lipid classes except phospholipids were quantitated fluorometrically in situ (Nicolosi et al.,
Phospholipids were eluted from the chromatoplates and quantitated colorimetrically using 0.02% malachite green according to the method of Chalvardjian and Rudnick (1970) in a Beckman Model 26 doublebeam spectrophotometer.

B. Standard Lipid Mixture and Thin-Layer Chromatography

Preparation of Lipid Standard

Highly purified (99+%) lipids were obtained as follows: cholesterol, L-(ω4)-dipalmitoyl lecithin (phospholipid), triolein (triacylglycerol), cholesteryl oleate, and palmitic acid from Applied Science Laboratories (State College, Pa.); and, squalene from K and K Laboratories, Inc. (Plainview, N.Y.). All compounds migrated as homogeneous spots on TLC chromatograms.

Twenty-five milligrams of each of the above reference standards were dissolved in 25.0 ml dichloromethane to give a final concentration of one microgram of each lipid per microliter of solution. This standard lipid solution was hydrogenated according to the previously mentioned procedure and stored under N2(g) at -35°C to -40°C until needed.

Thin-layer Chromatography

A detailed description of the solvent systems used in the lipid analyses has been previously published (Hojnacki and Smith, 1974). However, before the addition of solvents to the TLC tanks, the inner walls of the TLC chambers were lined with Whatman #1 filter paper to increase atmospheric saturation for even migration of lipid spots and solvent front. The TLC chambers used in the development of the chromatograms were equilibrated overnight with approximately 150 ml of their respective solvents.
Prior to use, the silica gel G chromatoplates #60 (EM Laboratories, Elmsford, N.Y.) were placed in the developing chamber containing solvent I (diethyl ether:glacial acetic acid:petroleum ether, 100:3:97) for pre-washing to move organic contaminants to the uppermost edge of the plates. After allowing the plate to air dry (30 min.), a very light pencil line was drawn across the absorbent side of the plate, 2.0 cm from the bottom to serve as a spotting line. The plate was then activated by heating 30-45 min. at 110°C.

Aortic samples and the lipid standard were spotted on the TLC plates according to the procedure described by Nicolosi et al. (1971).

After the plates were spotted, they were placed in solvent I and the solvent front was allowed to migrate 11 cm above the bottom of the plate. The plate was then removed from the chamber and allowed to air dry for 20-30 min. Next, the chromatoplate was placed in solvent II (3% diethyl ether in petroleum ether) in another chamber. Solvent II was allowed to migrate 18 cm above the bottom of the plate. This chromatogram was then air dried (20-30 min.) and sprayed twice to saturation with 0.001% Rhodamine 6G in 4.35% K$_2$HPO$_4$ buffer to visualize the lipid classes separated.

C. Fluorometric Scanning, Integration, and Calculation

Intensity of fluorescence from each lipid spot was measured in situ with a Turner Model 111 Filter Fluorometer and a Camag-Turner Model II Automatic TLC scanner. A low-pressure, mercury, far U.V. lamp with maximum emission at 250 nm was installed in combination with the primary filter, Corning 7-54 (Turner 110-811), transmittance 220-400 nm. A Turner 110-815 U.V. labile, absorbing filter (transmittance at 254-300 nm) was used in addition to the primary filter. The
secondary filter was a Turner 110-824 (color spec. #23A; max. transmittance 570 nm) in combination with a Turner #110-823L range extension filter (transmission 10%). A sensitivity adjustment of 10X was optimal.

Emitted fluorescence was plotted on a Barber-Colman "Chromocorder." The scanning direction was perpendicular to chromatographic resolution in order to scan all spots of the same class on the same recorder tracing.

Fluorescence-response peak areas for various concentrations of samples and standards were determined by triangulation and expressed in units of square millimeters. Quantitation of the sample was effected by comparing the peak area for each class with the standard curve for each lipid class derived from various quantities of standard spotted on the plate.

The accuracy and precision of this method has been reported by Nicolosi et al. (1971).

3. Non-Esterified Fatty Acid Profile Analysis

Once the excised aortic tissue was cleaned and stripped of adventitia, 10 mg of tissue from either the upper thoracic region or the celiac area was homogenized in 1.0 ml physiologically-buffered saline (pH 7.0). A 0.2 ml aliquot was taken for DNA analysis, and the remaining aliquot was used for the extraction of lipid by the method of Folch et al. (1957). The final lipid extract was dissolved in 10.0 ml dichloromethane: methanol (2:1, v/v). A 4.0 ml aliquot and 25 ul lipid standard were separated into lipid classes by thin-layer chromatography. After identification of the six lipid classes by comparison with the standard spots, the spot corresponding to free
fatty acids was eluted from the silica gel with diethyl ether into a round-bottom flask by the method of Goldbrick and Hirsch (1963). Diethyl ether was evaporated under a stream of N₂(g) at 30°C, and 0.50 ml of heptadecanoic acid solution (10 mg/ml CH₂Cl₂; Applied Science Laboratories, State College, Pa.) was added to the flask as internal standard along with 5.0 ml methanol:dichloromethane (6:1, v/v) for direct micro-methanolysis with BCl₃(g) by the method of Koes (1971).

Qualitative and quantitative GLC analyses were performed according to established procedures (Koes, 1971; McMullin et al., 1968). All gas-liquid chromatographic analyses were performed on a Barber-Colman Model 10 chromatograph with a strontium -90 argon ionization detector and a 6 ft X 0.25 in. siliconized (dichlorodimethyl silane) glass column packed with 12% ethylene glycol succinate on Anakrom A (80/90 mesh) (Analabs, North Haven, Conn.) and maintained at 187°C with 10 psi inlet pressure. Detector and flash heater temperatures were maintained at 237°C and 235°C, respectively, throughout the analyses. The column was calibrated with Applied Science (State College, Pa.) fatty acid standard K-108 and agreed with the stated composition data with a relative error less than 4% for major components. Standard fatty acid methyl esters showed a linear response over the range of sample sizes analyzed, and gas chromatographic peaks were identified by comparing retention times with reference standards. Areas of chromatographic peaks were determined by triangulation.

4. Transhydrogenation and Oxidative Phosphorylation in Arterial Tissue

A. Preparation of Tissue and Isolation of Mitochondria

Celiac cushions and upper thoracic aortas were removed immediately
following exsanguination and dissected free of adherent blood, and perivascular and adventitial tissue in 250 mM sucrose (2-4°C) supplemented with 0.5% bovine serum albumin (fatty acid poor, Fraction V; Sigma). Aortic tissue from eight pigeons was pooled in order to isolate sufficient mitochondria from the intima-media region. After aortas were stripped, they were placed in 20 ml isolation medium A (2-4°C) designed specifically to obtain and preserve functionally intact mitochondria. \[\text{Medium A: 250 mM sucrose, 100 mM KCl, 1 mM EDTA, 6 mM MgCl}_2, 25 \text{ mM Tris-HCl buffer (pH 7.4), (3:1:1:1:1, v/v/v/v/v)}\]. The tissue was subsequently minced into small pieces (ca. 1 mm\(^2\)). (All solutions were prepared using 10 megohm water, and all glassware was rinsed three times with this water.)

Tissue from the celiac bifurcation (approx. 50 mg wet tissue wgt.) was digested with elastase and collagenase (Morrison et al., 1970) in order to provide maximal yields of mitochondria from smooth muscle cells of the muscular foci. The elastase concentration (EC 3.4.4.7; Worthington Biochemical Corp., N.J.) used was 5 units/mg. wet tissue wgt. prepared in 1.0 ml HBSS with glucose. Digestion was carried out for 1-2 hrs at 34°C in a Dubnoff Shaking Water Bath. The pH was maintained at 7.0 with 0.17M NaHCO\(_3\). After 2 hrs, collagenase (EC 3.4.4.19; Worthington Biochemical Corp.) was added directly to the mixture (containing elastase) for 1 hr. (15 units/mg. wet tissue wgt.) under the same incubation conditions. Subsequent to the completed digestion, the preparations were placed in 10 ml isolation medium A and centrifuged twice at 700 X g for 10 min to remove nuclei and debris (2-4°C). The resulting supernatant was centrifuged twice at 10,000 X g for 10 min to yield a mitochondrial fraction which was
washed and re-suspended in 2 ml of reaction assay medium containing 250 mM mannitol, 1 mM EDTA, 20 mM KCl, 6 mM MgCl₂, and 25 mM Tris-HCl buffer (pH 7.2), (3:1:1:1:1, v/v/v/v/v). Argon was flushed very slowly into the reaction medium for approximately 30 sec to remove dissolved oxygen which can cause cavitation of mitochondria and render them susceptible to disruption (Kalra and Brodie, 1973).

Since larger amounts of tissue were available from the upper thoracic regions of the aorta than the celiac cushions, enzymatic digestion was not required to maximize the mitochondrial yields. After mincing the aortic tissue, mitochondrial fractions were obtained by placing the tissue in 30 ml isolation medium A and homogenizing in a VirTis Homogenizer for 20 sec at 24,000 rpm at 2-4°C with a macroshaft assembly. The homogenate was filtered through one layer of cheesecloth. Residual tissue was re-suspended in isolation medium A and re-homogenized by the procedure described above. The filtrates were combined and centrifuged twice at 700 X g for 15 minutes to remove nuclei and debris. The resulting supernatant was then used for the isolation of mitochondria. The supernatant was centrifuged twice at 10,000 X g for 10 minutes to yield a mitochondrial pellet which was washed and re-suspended in 2 ml of reaction assay medium. Argon was also flushed through this reaction mixture.

The direct addition of exogenous cytochrome c₁ (Type XIV from Pigeon Heart; Sigma, St. Louis, Mo.) (0.3 mg/mg mito. protein) to the mitochondrial fraction which was suspended in the reaction assay medium was necessary in order to observe high respiratory control since there can be loss of endogenous cytochrome c₁ during the homogenization (Hedman, 1965; Kalra and Brodie, 1973; 1974).

In order to determine if the mitochondria were prepared without
damage, an index was needed to determine whether they remained functionally intact during the isolation procedures. Once this was established, P/O ratios were subsequently determined. Aortic mitochondria from these preparations were considered to be reasonably good if they exhibited an acceptor (respiratory) control ratio (ACR) of 2.0±0.1 with succinate as substrate. Mitochondria isolated from aortic tissue in isolation medium A repeatedly exhibited an ACR of 2.0±0.1. Also, it is noteworthy that the use of other isolation media as reported by Kalra and Brodie (1973, 1974), Morrison et al. (1973), and Simard-Duquesne (1969) did not prove satisfactory in maintaining functionally intact mitochondria from pigeon aortic tissue (ACRs ranged from 1.3-1.5 with succinate as substrate).

The two methods for the isolation of mitochondria yielded preparations having similar P/O ratios with \( \beta \)-hydroxybutyrate as substrate. A 160 mg pool of aortic tissue (WC upper thoracic aortas, 4-6 weeks of age) was minced (ca. 1 mm\(^2\)) in isolation medium A and separated into 4 separate portions each weighing 40 mg. Two portions were subjected to enzymatic digestion to isolate mitochondria, and the mitochondria exhibited P/O ratios of 2.02 and 2.12. The other two portions were treated by the VirTis homogenization procedure and exhibited P/O ratios of 2.09 and 1.98.

Mitochondrial protein was determined by the method of Lowry et al. (1951) after mitochondrial preparations were washed free of albumin. Bovine serum albumin (Fraction V; Sigma) was used as a standard.

B. Preparation of Aortic Elementary Transfer Particles (ETP\(_A\))

In order to examine the energy-dependent (ATP) reduction of NADP\(^+\) by NADH, elementary transfer particles were prepared by a method
developed originally by Low and Vallin (1963) with the following modifications:

Once aortic mitochondria were prepared and examined for functional capability (ACR $2.0^{\pm}0.1$ with succinate), they were then re-suspended in a 2.5 ml 250 mM sucrose solution B supplemented with 15 mM MgCl$_2$, 20 mM Tris–HCl (pH 7.2), and 10 mM KCl (3:1:1:1, v/v/v/v). Argon (g) was slowly bubbled through this suspension for approximately 30 sec. Then it was treated with high frequency sonic oscillation with a Tekmar Sonic Oscillator at low speed (setting #50 on SE Powerstat) for 5 min at 1 min intervals (temp. 2-4°C; pH maintained at 7.4 with 0.05N NaOH). This homogenate was centrifuged at 9,500 X g for 10 min. The supernatant was re-centrifuged at 100,000 X g for 10 min, and the pellet was re-suspended in 5 ml of 250 mM sucrose solution. This suspension was subjected to homogenization – sonication once again at pH 7.4 to produce a uniform dispersion of spherical A-particles in the homogenate. The suspension was then centrifuged at 100,000 X g for 40 min to give a final sediment of particles. After protein analysis of the ETP$_A$'s, the suspension was adjusted to a final concentration of 0.75 mg ETP$_A$ protein per ml by adding sucrose solution. The yield of particles corresponded to 65-75% of the protein in the isolated aortic mitochondrial fraction (1 mg mito. protein/60 mg wet tissue wt).

C. Respirometry: Determination of Acceptor Control Ratios by Polarographic Analysis

Respiration of mitochondria (0.35-0.40 mg protein) in state 3 (oxygen consumption with ADP) and state 4 (oxygen consumption without ADP) was measured at 30°C in a total volume of 1.75 ml using a Clark oxygen electrode built to accommodate micro-reaction chambers. These
experiments were performed periodically to examine the functional integrity of the mitochondrial suspensions. Oxygen uptake was monitored in the microchamber containing:

- 0.25 ml 10 mM KH$_2$PO$_4$ buffer (pH 7.4)
- 0.10 ml 250 mM mannitol
- 0.10 ml 40 mM KCl
- 0.15 ml 20 mM NaF
- 0.15 ml 5 mM iodoacetate
- 0.25 ml 6 mM MgCl$_2$
- 0.35 ml 10 mM succinate

Acceptor control ratios (ACR) were determined by the method of Chance (1959) and Estabrook (1967) with the following modifications: 0.25 mg cyt c$_1$/mg mitochondrial protein (Pigeon Heart, Type XIV, Sigma Chemical Co.) was added to the reaction assay medium; and, after the initial rate was established with succinate (state 4), 0.40 ml 1.0 mM ADP / 20 mM Tris-HCl (pH 7.2) was added to drive mitochondria into state 3 respiration.

D. Respirometry: Evaluation of Coupled Oxidative Phosphorylation by P/O Ratios

Oxidative phosphorylation was studied by oxygen polarigraphy in a Gilson Polarograph-Oxygraph K-IC equipped with micro-cell attachment for volumes approaching 2.00 ml. A Clark oxygen electrode OX-15250 (Rainin Instruments, Boston, Mass.) was used to monitor oxygen uptake at 30°C. A water jacketed cell was used with the oxygraph in order to minimize air diffusion. Also, a capillary bore stopper was employed to insure venting of the cell thereby equilizing the pressure inside and outside the cell. This reduces diffusion of
oxygen into the cell to a negligible value (Rainin Instruments, personal communication). Polarizing voltage for the Clark electrode was maintained at 0.8 volts. A magnetic stirrer was placed under the reaction microchamber in order to avoid the formation of interfering oxygen concentration gradients.

Optimal concentrations were used in the incubation reaction mixture as follows:

<table>
<thead>
<tr>
<th>Volume</th>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.30 ml</td>
<td>10.0 mM</td>
<td>KH$_2$PO$_4$ buffer (pH 7.2)</td>
</tr>
<tr>
<td>0.25 ml</td>
<td>20.0 mM</td>
<td>β-hydroxybutyrate or succinate</td>
</tr>
<tr>
<td>0.30 ml</td>
<td>10.0 mM</td>
<td>ADP (added to rxn. mixture in 0.10 ml aliquots)</td>
</tr>
<tr>
<td>0.05 ml</td>
<td>0.15 mg/mL</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>0.30 mg</td>
<td></td>
<td>cyt c$_1$/mg. mito. protein</td>
</tr>
</tbody>
</table>

The reaction was started with 1.0 ml mitochondrial suspension (1.0 mg mito. protein/ml reaction medium). To inhibit substrate level phosphorylation, all reactions were carried out in the presence of 0.05 ml 20.0 mM NaF and 0.05 ml 5 mM iodoacetate. Total volume of reaction medium in the microchamber was 2.0 ml. The reaction was allowed to proceed for 1 hr, and then terminated by the addition of 0.10 ml 10% trichloroacetic acid. Aliquots were taken before and after the reaction to analyze for inorganic phosphate (P$_i$). Solutions were centrifuged at 700 X g for 10 min, and the supernatants were analyzed for P$_i$ by the method of Lindberg and Ernster (1956) with 1.0 mM KH$_2$PO$_4$ as a reference standard. Results of the P$_i$ analyses were expressed as umole P$_i$ utilized/hr/mg mito. protein.

Oxygen uptake was calculated by the method of Chance and Williams (1955) as modified by Estabrook (1967) from information based on its solubility (Bunsen) coefficient in solution at 1 atm at 30°C.
Results were expressed as umoles \( O_2/hr/mg \) mito. protein. The P/O ratio was then calculated from these results.

\( \epsilon \)-hydroxybutyrate was chosen as a substrate since it can produce the highest P/O ratio by its electrons entering the respiratory chain prior to the first coupling site (maximum theoretical P/O ratio is 3). Succinate was chosen as a second substrate (max. theoretical P/O ratio is 2) since it can alter the NADH/NAD\(^+\) ratio by reverse electron flow and, thereby, affect respiration and the synthetic rate of fatty acids in the aorta (Whereat, 1967). Whereat (1967) has shown that the rate of \( O_2 \) consumption with succinate is 3-5 times faster than with any other Krebs cycle intermediate or glucose, and cholesterol-induced atherosclerotic intima-media preparations had approximately 2.5 times greater \( O_2 \) consumption than non-atherosclerotic controls using succinate. These unique responses of the aorta to succinate are thought to be related to a loss of acceptor control caused by atherosclerosis. Since succinate appears to be a key metabolite in relating oxidative metabolism to lipid synthesis, it was thought advantageous to compare P/O ratios between atherosclerosis-susceptible and -resistant aortic foci using this substrate in order to ascertain whether there is a difference in coupling of respiratory-chain phosphorylation prior to the development of the early lesion in the susceptible foci.

Finally, it was also thought advantageous to determine the maximal amount of uncoupling which could be detected with this experimental system. Mitochondrial preparations from six week old aortas were incubated in standard reaction medium in the presence of the uncoupling agent dicumarol (10mM). Uncoupling agents increase
oxygen uptake in aortic tissue (Murray et al., 1968), thereby decreasing P/O ratios. The results in Table 1 (page 30) show the P/O ratios were consistently lower in dicumarol-treated mitochondria compared to non-treated controls for both celiac cushions and thoracic segments of the aorta. Further comparison shows that the decreased P/O ratios in the presence of dicumarol were due to maximal stimulation of oxygen uptake with slight increases in inorganic phosphate utilization. It appears from these experiments that a real phenomena associated with mitochondrial oxidative phosphorylation and oxygen uptake has been demonstrated.

E. Energy-linked Transhydrogenation in Aortic Tissue: Fluorometric Scanning of \( \text{NAD}^+ \) and \( \text{NADP}^+ \) Reduction

The energy-dependent (ATP) reduction of \( \text{NADP}^+ \) by \( \text{NADH} \) was examined by the method originally described by Danielson and Ernster (1963). The modified incubation reaction mixture consisted of:

- 0.15 ml 6 mM MgCl₂
- 0.10 ml 250 mM sucrose
- 0.10 ml 50 mM Tris-HCl buffer (pH 7.4)
- 0.15 ml 60 mM ethanol
- 0.25 mg alcohol dehydrogenase (Calbiochem)
- 0.15 ml 1 mM rotenone (to block aerobic oxidation of \( \text{NADH} \))
- 0.10 ml 0.15 mM \( \cdot \)-chloromercuribenzoate
- 1.00 ml WC or SR \( \text{ETP}_A \) (0.75 mg mito. protein/ml)
- 0.05 ml 0.02 mM \( \text{NAD}^+ \)
- 0.10 ml 0.20 mM \( \text{NADP}^+ \)
- 0.35 ml 2 mM ATP

The final volume was maintained at 2.25 ml. The reaction was followed...
at room temperature with a Turner Scanning Fluorometer Model 111
coupled to a Barber-Colman recorder to visualize NAD⁺ reduction
and the ATP-dependent reduction of NADP⁺ by NADH. The primary
filter used in the experiments was a #7-60 (Turner 110-811) for
excitation at 365 nm, and secondary filters Kodak Wratten 2A
(Turner 110-816) and Kodak Wratten 47B (Turner 110-813) for emis­
sion between 435-450 nm. (2A was placed closest to the lamp.)

In order to characterize NADH transhydrogenase activity from
aortic tissue, preliminary experiments revealed that transhydro­
genase activity from WC and SR aortas was completely inhibited by
10 mM diacetyl. It has been documented to inhibit mitochondrial
transhydrogenase activity in other systems (Hatefi and Stiggall,
1976). In addition, transhydrogenation from WC celiac foci was
not affected by the uncoupling agent, dicumarol (1 mM) which can
also serve as an electron transfer inhibitor; whereas, ATP-dependent
transhydrogenase activity of 1-day to 6-month old WC thoracic aortas,
and SR celiac foci and thoracic aortas was completely inhibited by
dicumarol.

Since aortic elementary transfer particles (ETPA) were prepared
by sonication of mitochondria, it is possible that the observed lack
of regulation by ATP of NADP⁺ reduction via NADH in WC celiac foci
was either due to the solubilization of the enzyme or the enzyme was
no longer membrane-bound due to the isolation methods used to pro­
file transhydrogenation. Preliminary experiments revealed, however,
that "intact" mitochondria from six-week old WC celiac foci which
exhibited high acceptor control with succinate showed NADP⁺ reduction
by NADH in the absence of ATP. This indicates that the enzyme was
membrane-bound and not solubilized during the preparation of the ETP$_A$s from the mitochondria.
Figure 1: DNA release as a function of Homogenization-Proteolytic Digestion. Each point represents the amount of DNA in a 0.15 ml aliquot taken from 1.5 ml homogenate containing 15.5 mg wet tissue weight of six-month old WC aortas prepared in physiologically-buffered saline.
Table 1: Rates of Phosphorous and Oxygen Uptake of Dicumarol-
Treated Pigeon Aortic Mitochondria

<table>
<thead>
<tr>
<th></th>
<th>White Carneau&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Show Racer&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P/O</td>
<td>P&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Celiac</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dicumarol</td>
<td>0.85</td>
<td>2.55</td>
</tr>
<tr>
<td>Dicumarol</td>
<td>0.77</td>
<td>2.67</td>
</tr>
<tr>
<td>Control</td>
<td>2.35</td>
<td>2.00</td>
</tr>
<tr>
<td>Thoracic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dicumarol</td>
<td>0.75</td>
<td>1.74</td>
</tr>
<tr>
<td>Dicumarol</td>
<td>0.65</td>
<td>1.49</td>
</tr>
<tr>
<td>Control</td>
<td>2.43</td>
<td>1.01</td>
</tr>
</tbody>
</table>

a. Six-week old WC and SR aortas were used in these experiments. 

β-hydroxybutyrate was used as a substrate for the controls and dicumarol-treated preparations.

b. P: μmoles P<sub>i</sub> utilized/hr/mg mito. protein

c. O: μmoles O consumed/hr/mg mito. protein
EXPERIMENTAL DESIGN

This study was designed as follows:

<table>
<thead>
<tr>
<th>Age</th>
<th>White Carneau</th>
<th>Show Racer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>celiac focus</td>
<td>upper thoracic</td>
</tr>
<tr>
<td>1 day</td>
<td>w</td>
<td>y, z w</td>
</tr>
<tr>
<td>6 weeks</td>
<td>w, x, y, z</td>
<td>w, x, y, z</td>
</tr>
<tr>
<td>12 weeks</td>
<td>w, y, z</td>
<td>w, y, z</td>
</tr>
<tr>
<td>6 months</td>
<td>w, x, y, z</td>
<td>w, x, y, z</td>
</tr>
</tbody>
</table>

Each w represents the total and six individual lipid classes (ug/ug DNA) for one experimental unit. Each x represents the total and individual free fatty acids (ug/ug DNA) for one experimental unit. Each y represents the NADH transhydrogenation profile, and z represents the P/0 ratio (including μmoles P₄ utilized/hr/mg protein and μmoles O consumed/hr/mg protein) for the experimental unit. The entire thoracic aortas including celiac bifurcations were pooled for the one-day analyses during the energy metabolism studies in order to isolate enough aortic mitochondria.

Four separate replicates of celiac and upper thoracic portions of the aortas were performed for all lipid analyses for each breed at each age. Three to five separate replicates were performed to calculate P/O ratios for each breed at each age, and two separate replicates were performed to examine NADH transhydrogenation for each breed at each age.
All lipid analyses and P/O ratios derived from celiac cushions and upper thoracic aortas for WC and SR pigeons were compared statistically with analyses of variance (Neter and Wasserman, 1974). The results of the lipid and energy metabolism studies including transhydrogenation profiles were compared in three ways: corresponding aortic sites between breeds at each age, two sites within each breed at each age, and each site within each breed at various ages.
RESULTS

1. Quantitative Lipid Analysis

A. Total Lipid Composition

There were no significant sex-related differences between the amounts of any lipid class in WC or SR pigeon aortas at either site in any of the four age groups studied. Therefore, values for both sexes were grouped together for presentation in Tables 2, 4, 6 and 7 (pages 37, 39, 41, 42). (An equal number of males and females were used in each case.)

Age trends and breed differences

Total lipid in WC and SR celiac foci increased significantly at each successive age studied after one-day (Tables 2 and 3, pages 37, 38). However, WC celiac foci had significantly more total lipid at 6 weeks and 6 months of age than SR celiac foci, the largest difference being at 6 months of age (Figure 2, page 45). A similar increasing trend in total lipid was observed in thoracic aortas of both breeds, but the amount of increase was much less, and there were no major differences between breeds (Figure 2, page 45; Tables 4 and 5, pages 39, 40) in total lipid or in any individual lipid class.

Although cholesterol was the predominant lipid in both WC and SR celiac foci at 1 day and showed a marked increase by 6 weeks, it was only at 6 months of age that there was a major difference between the WC and SR foci (Figure 3, page 47) with WC having significantly more than SR (Table 3, page 38). By this age, however, cholesterol comprised
a much smaller proportion of the total lipid in each breed. Non-
esterified fatty acid (NEFA), the other major lipid class in 1-day WC
foci, was much greater than in SR foci, and underwent a dramatic increase
by 6 weeks to plateau and remain significantly greater than SR celiac
NEFA (Figure 4, page 49; Table 3, page 38) despite an increase in the
latter at each succeeding age.

From 1 day to 12 weeks of age, there was little difference between
breeds in the general trend toward increased amounts of triacylglycerols
(Figure 5, page 51), cholesteryl esters (Figure 6, page 53), phos-
pholipids (Figure 7, page 55), and squalene (Figure 8, page 57) in
the celiac foci. After 12 weeks, however, absolute amounts of all
lipid classes except squalene were significantly (p < 0.05) greater in
WC celiac foci than SR celiac foci (Tables 2 and 3, pages 37, 38).
A breed difference in squalene content at celiac foci was detected
only at 12 weeks of age (Table 2 and 3, pages 37, 38).

Finally, it is noteworthy that triacylglycerols and cholesteryl
esters are present in proportions similar to cholesterol and free
fatty acids in WC celiac foci by 6 months while phospholipids and
squalene comprise the majority of lipid in both breeds at this age
(Table 2, page 37).

Aortic Site Differences

Celiac foci (Table 2, page 37) have more total lipid than
thoracic segments (Table 4, page 39) by 6 weeks in WC and by 12 weeks
in SR (Table 3, page 38).

Cholesterol was significantly greater in WC celiac foci than
thoracic aortas at 12 weeks and 6 months of age. Similar differences
were seen in SR at 6 weeks and 6 months of age. Although non-esterified
fatty acids were significantly greater in WC celiac foci than in thoracic aortas at 1 day and subsequent ages (Table 3, page 38) a similar difference was not observed in the SR until 6 months of age. It is noteworthy that an increase in cholesteryl esters was seen at 12 weeks in SR celiac foci; however, in contrast to WC foci in which cholesteryl esters increased dramatically by 6 months, levels of cholesteryl esters (as well as of triacylglycerols) in SR celiac foci remained essentially the same and approximated those in WC and SR thoracic aortas at 6 months of maturation (Figure 6, page 53). Finally, phospholipid and squalene accumulation (Figures 7 and 8, pages 55, 57) is more prevalent in the celiac foci of both breeds than in thoracic areas of the aorta at 12 weeks and 6 months of age.

B. Profile of Non-Esterified Fatty Acids

Since the earliest major breed difference in lipid composition between the celiac foci was seen in NEFA at 6 weeks of age, the nature of the fatty acids were examined at this age and at 6 months.

Comparison of total amounts of individual non-esterified fatty acids revealed that 6 week-old WC celiac foci contained significantly more fatty acids than SR celiac foci. This was primarily due to the larger amounts of palmitate, stearate, oleate, and linoleate in WC compared to the SR, although the relative proportions of these free fatty acids were generally the same except for oleate between the WC and SR (Table 6, page 41).

Major age-related trends from 6 weeks to 6 months of age in WC celiac foci and upper thoracic aortas were increased amounts and proportions of shorter-chain fatty acids (myristic and myristoleic acids) and saturated fatty acids at the expense of unsaturated free
fatty acids (Tables 6 and 7, pages 41, 42). This is especially significant since WC celiac foci had greater quantities of unsaturated fatty acids than SR celiac foci at 6 weeks of age. Contrary to these age-related trends in WC, absolute amounts and proportions of unsaturated fatty acids increased during this age period in SR celiac foci and upper thoracic aortas while the total amounts in the SR foci more than doubled. Particularly noteworthy is the striking decrease in the unsaturated to saturated and the polyunsaturated to saturated ratios at both WC aortic sites as a function of age (Table 8, page 43). The opposite pattern occurs in the SR.
<table>
<thead>
<tr>
<th></th>
<th>Cholesterol</th>
<th>Non-Esterified Fatty Acids</th>
<th>Triglycerols</th>
<th>Cholesterol Ester</th>
<th>Phospholipids</th>
<th>Squalene</th>
<th>Total Lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>WC 1 day</td>
<td>0.32 ± 0.03 (29.4)</td>
<td>0.21 ± 0.03 (15.3)</td>
<td>0.04 ± 0.01 (5.7)</td>
<td>0.16 ± 0.02 (14.7)</td>
<td>0.13 ± 0.01 (11.9)</td>
<td>0.23 ± 0.04 (21.1)</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>SR 1 day</td>
<td>0.34 ± 0.05 (30.9)</td>
<td>0.05 ± 0.02 (4.5)</td>
<td>0.06 ± 0.01 (5.5)</td>
<td>0.16 ± 0.01 (14.5)</td>
<td>0.28 ± 0.01 (25.5)</td>
<td>0.21 ± 0.04 (19.1)</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>WC 6 weeks</td>
<td>3.38 ± 0.87 (20.9)</td>
<td>5.56 ± 0.44 (34.3)</td>
<td>2.33 ± 0.43 (14.4)</td>
<td>0.31 ± 0.05 (1.9)</td>
<td>1.96 ± 0.35 (12.1)</td>
<td>2.65 ± 0.36 (16.4)</td>
<td>16.2 ± 0.8</td>
</tr>
<tr>
<td>SR 6 weeks</td>
<td>2.04 ± 0.28 (23.3)</td>
<td>0.63 ± 0.14 (7.2)</td>
<td>0.99 ± 0.01 (11.2)</td>
<td>0.44 ± 0.09 (5.0)</td>
<td>2.06 ± 0.31 (23.4)</td>
<td>2.65 ± 0.28 (30.0)</td>
<td>8.8 ± 0.4</td>
</tr>
<tr>
<td>WC 12 weeks</td>
<td>2.53 ± 0.25 (11.2)</td>
<td>4.39 ± 0.83 (20.4)</td>
<td>4.14 ± 0.74 (18.4)</td>
<td>1.97 ± 0.17 (8.8)</td>
<td>3.49 ± 0.70 (15.5)</td>
<td>5.79 ± 0.42 (25.7)</td>
<td>22.5 ± 1.5</td>
</tr>
<tr>
<td>SR 12 weeks</td>
<td>1.03 ± 0.19 (7.0)</td>
<td>1.26 ± 0.23 (8.5)</td>
<td>2.56 ± 0.61 (17.2)</td>
<td>2.00 ± 0.37 (13.5)</td>
<td>3.89 ± 0.63 (26.3)</td>
<td>4.07 ± 0.16 (27.5)</td>
<td>14.8 ± 2.3</td>
</tr>
<tr>
<td>WC 6 months</td>
<td>7.49 ± 0.59 (11.6)</td>
<td>6.01 ± 0.40 (9.5)</td>
<td>7.82 ± 1.25 (12.4)</td>
<td>6.91 ± 0.47 (10.9)</td>
<td>14.50 ± 2.40 (22.9)</td>
<td>20.54 ± 3.19 (32.5)</td>
<td>63.3 ± 4.4</td>
</tr>
<tr>
<td>SR 6 months</td>
<td>1.57 ± 0.08 (4.6)</td>
<td>3.13 ± 0.55 (9.3)</td>
<td>2.03 ± 0.05 (6.0)</td>
<td>1.44 ± 0.30 (4.3)</td>
<td>7.75 ± 1.37 (22.9)</td>
<td>17.89 ± 3.87 (52.9)</td>
<td>34.8 ± 4.0</td>
</tr>
</tbody>
</table>

a. Values expressed as ug lipid/ug DNA ± SEM. Each value represents the mean from analyses of 4 tissue pools of at least 2 birds per pool. Statistical comparisons described in Table 3 (page 35).

b. Values in parenthesis are corresponding relative percentages of the total lipid.
TABLE 3: STATISTICAL ANALYSIS OF LIPID DATA PRESENTED IN TABLE X FOR WHITE CANDLA'S (WC) AND SHOW RACER (SR) CELIAC FOI.

<table>
<thead>
<tr>
<th></th>
<th>Age Comparisons</th>
<th>Breed Comparisons</th>
<th>Aortic Site Comparisons (Tables 2 and 4)</th>
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<td>1 day-6 wks</td>
<td>6 wks-12 wks</td>
<td>6 mos</td>
</tr>
<tr>
<td></td>
<td>6 wks</td>
<td>12 wks</td>
<td>6 mos</td>
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<td></td>
<td>6 mos</td>
<td></td>
<td></td>
</tr>
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<td>cholesterol</td>
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</tr>
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<td>S</td>
<td>NS</td>
<td>S</td>
</tr>
<tr>
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<td>S</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>NEFA</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>WC</td>
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<td>NS</td>
<td>NS</td>
</tr>
<tr>
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<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>TG</td>
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<td></td>
</tr>
<tr>
<td>WC</td>
<td>S</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
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<td>S</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CE</td>
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<td></td>
</tr>
<tr>
<td>WC</td>
<td>NS</td>
<td>S</td>
<td>NS</td>
</tr>
<tr>
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<td>NS</td>
<td>S</td>
<td>NS</td>
</tr>
<tr>
<td>phospho-lipids</td>
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</tr>
<tr>
<td>WC</td>
<td>S</td>
<td>NS</td>
<td>S</td>
</tr>
<tr>
<td>SR</td>
<td>S</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>squalene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WC</td>
<td>S</td>
<td>S</td>
<td>NS</td>
</tr>
<tr>
<td>SR</td>
<td>S</td>
<td>S</td>
<td>NS</td>
</tr>
<tr>
<td>Total Lipids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WC</td>
<td>S</td>
<td>S</td>
<td>NS</td>
</tr>
<tr>
<td>SR</td>
<td>S</td>
<td>S</td>
<td>NS</td>
</tr>
</tbody>
</table>

S = significantly different (p < 0.05)
NS = not significantly different
TABLE 4: LIPID CONTENT OF UPPER THORACIC AORTAS AS A FUNCTION OF AGE IN WHITE CASHEAU (WC) AND SHON RACER (SR) PIGEONS

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol</th>
<th>Non-Esterified Fatty Acids</th>
<th>Triacylglycerols</th>
<th>Cholesteryl Esters</th>
<th>Phospholipids</th>
<th>Squalene</th>
<th>Total Lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WC</td>
<td>0.18 ± 0.02* (14.8)b</td>
<td>0.26 ± 0.02 (21.3)</td>
<td>0.26 ± 0.04 (21.3)</td>
<td>0.24 ± 0.04 (19.7)</td>
<td>0.21 ± 0.05 (17.2)</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>SR</td>
<td>0.19 ± 0.07 (15.4)</td>
<td>0.29 ± 0.06 (23.6)</td>
<td>0.18 ± 0.05 (14.6)</td>
<td>0.22 ± 0.04 (17.9)</td>
<td>0.29 ± 0.09 (23.6)</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>1 day</td>
<td>WC</td>
<td>1.21 ± 0.20 (16.4)</td>
<td>1.39 ± 0.18 (14.5)</td>
<td>0.19 ± 0.01 (2.6)</td>
<td>1.77 ± 0.34 (24.1)</td>
<td>1.73 ± 0.36 (23.5)</td>
<td>7.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>SR</td>
<td>0.65 ± 0.13 (11.4)</td>
<td>1.45 ± 0.10 (25.4)</td>
<td>0.52 ± 0.09 (9.2)</td>
<td>1.23 ± 0.19 (21.6)</td>
<td>1.49 ± 0.15 (26.1)</td>
<td>5.7 ± 0.4</td>
</tr>
<tr>
<td>5 weeks</td>
<td>WC</td>
<td>0.66 ± 0.20 (10.2)</td>
<td>0.29 ± 0.02 (4.5)</td>
<td>0.49 ± 0.13 (7.4)</td>
<td>1.59 ± 0.38 (24.6)</td>
<td>1.70 ± 0.14 (26.2)</td>
<td>6.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>SR</td>
<td>0.44 ± 0.08 (8.4)</td>
<td>1.58 ± 0.48 (30.0)</td>
<td>0.23 ± 0.08 (4.4)</td>
<td>0.51 ± 0.17 (9.7)</td>
<td>1.62 ± 0.17 (30.6)</td>
<td>5.3 ± 0.7</td>
</tr>
<tr>
<td>12 weeks</td>
<td>WC</td>
<td>0.50 ± 0.15 (4.4)</td>
<td>1.62 ± 0.15 (14.2)</td>
<td>0.24 ± 0.16 (10.9)</td>
<td>1.46 ± 0.28 (12.8)</td>
<td>3.59 ± 0.52 (31.6)</td>
<td>11.4 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>SR</td>
<td>0.09 ± 0.01 (1.0)</td>
<td>1.23 ± 0.06 (13.9)</td>
<td>0.00 ± 0.05 (11.3)</td>
<td>1.04 ± 0.04 (11.8)</td>
<td>3.04 ± 0.14 (34.6)</td>
<td>8.6 ± 0.4</td>
</tr>
</tbody>
</table>

a. Values express* as ug lipid/ug DNA ± SEM. Each value represents the mean from separate analyses of 4 tissue pools consisting of at least 2 birds per pool. Statistical comparisons described in Table 5 (page 4b).

b. Values in parenthesis are corresponding relative percentages of the total lipid.

-
<table>
<thead>
<tr>
<th></th>
<th>Age Comparisons</th>
<th>Breed Comparisons</th>
</tr>
</thead>
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<td></td>
<td>1 day- 6 wks- 6 wks</td>
<td>12 wks- 6 mo.</td>
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<td>SR NS NS NS NS</td>
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<td></td>
<td>CE</td>
<td>WC NS NS NS NS NS NS NS NS NS</td>
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<tr>
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<td>phos-</td>
<td>WC S NS NS NS NS NS NS NS NS</td>
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<td>lipids</td>
<td>phos-</td>
<td>WC S NS S S NS NS NS NS NS</td>
</tr>
<tr>
<td>squalene</td>
<td>squalene</td>
<td>WC S NS S S NS NS NS NS NS</td>
</tr>
<tr>
<td>Total Lipids</td>
<td>Total</td>
<td>WC S NS S S NS NS NS NS NS</td>
</tr>
</tbody>
</table>

S = significantly different (P < 0.05)
NS = not significantly different
## TABLE 6: NON-ESTERIFIED FATTY ACIDS IN WHITE CARNEAX (WC) AND SHOW BACER (SR) AORTAS AT SIX WEEKS OF AGE

<table>
<thead>
<tr>
<th></th>
<th>WC</th>
<th>SR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>celiac cushions upper thoracic</td>
<td>celiac cushions upper thoracic</td>
</tr>
<tr>
<td>14:0</td>
<td>trace (&lt;1%)</td>
<td>trace (&lt;1%)</td>
</tr>
<tr>
<td>14:1</td>
<td>trace (&lt;1%)</td>
<td>trace (&lt;1%)</td>
</tr>
<tr>
<td>16:0</td>
<td>1.65 ± 0.27b,c,i (33.2)</td>
<td>0.18 ± 0.02i (21.7)</td>
</tr>
<tr>
<td>18:0</td>
<td>0.69 ± 0.11d,i (14.4)</td>
<td>0.09 ± 0.02d (10.9)</td>
</tr>
<tr>
<td>18:1</td>
<td>2.49 ± 0.16e,k (41.7)</td>
<td>0.46 ± 0.08h,k (56.7)</td>
</tr>
<tr>
<td>18:2</td>
<td>0.63 ± 0.26f,i (10.5)</td>
<td>0.07 ± 0.01f (6.1)</td>
</tr>
<tr>
<td>18:3</td>
<td>trace (&lt;1%)</td>
<td>0.03 ± 0.02 (2.6)</td>
</tr>
<tr>
<td>20:3</td>
<td>trace (&lt;1%)</td>
<td>trace (&lt;1%)</td>
</tr>
<tr>
<td>20:4</td>
<td>trace (&lt;1%)</td>
<td>trace (&lt;1%)</td>
</tr>
<tr>
<td>Total</td>
<td>5.47 ± 1.50g,m</td>
<td>0.85 ± 0.18m</td>
</tr>
</tbody>
</table>

- a. values in parenthesis are relative percentages.
- b. ug lipid/ug DNA ± SEM. Each value represents the mean from separate analyses of 4 tissue pools of at least 2 birds per pool.
- c-n. values with the same superscript are significantly different (p<0.05).
- c-h. differences between breeds.
- i-n. differences between aortic sites.

### Saturated Fatty Acids
- 14:0 myristic acid
- 16:0 palmitic acid
- 18:0 stearic acid

### Unsaturated Fatty Acids
- 14:1 myristoleic acid
- 18:1 oleic acid
- 18:2 linoleic acid
- 18:3 linolenic acid
- 20:3 eicosatrienoic acid
- 20:4 arachidonic acid
### TABLE 7: NON-ESTERIFIED FATTY ACIDS IN WHITE CARNEAU (WC) AND SHOW RACER (SR) AORTAS AT SIX MONTHS OF AGE

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</thead>
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<td>upper thoracic</td>
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<tr>
<td></td>
<td>(ug lipid/ug DNA ± SEM)</td>
<td>(ug lipid/ug DNA ± SEM)</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>(Relative percentages)</td>
<td>(Relative percentages)</td>
</tr>
<tr>
<td>14:0</td>
<td>0.07 ± 0.03 (1.6)</td>
<td>trace (1%)</td>
</tr>
<tr>
<td></td>
<td>0.06 ± 0.02 (5.1)</td>
<td>trace (1%)</td>
</tr>
<tr>
<td>14:1</td>
<td>0.07 ± 0.03 (1.6)</td>
<td>trace (1%)</td>
</tr>
<tr>
<td></td>
<td>0.07 ± 0.03 (4.9)</td>
<td>trace (1%)</td>
</tr>
<tr>
<td>16:0</td>
<td>2.40 ± 0.15c,k (52.0)</td>
<td>0.48 ± 0.05c,o (22.2)</td>
</tr>
<tr>
<td></td>
<td>0.72 ± 0.12g,k (56.7)</td>
<td>0.23 ± 0.01g (21.4)</td>
</tr>
<tr>
<td>18:0</td>
<td>1.39 ± 0.29d,1 (29.0)</td>
<td>0.34 ± 0.06h,1 (27.0)</td>
</tr>
<tr>
<td></td>
<td>0.21 ± 0.05d (10.1)</td>
<td>0.09 ± 0.01h (8.6)</td>
</tr>
<tr>
<td>18:1</td>
<td>0.69 ± 0.15m (14.3)</td>
<td>0.08 ± 0.03d,i,m (5.7)</td>
</tr>
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<td>0.94 ± 0.16p (42.6)</td>
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<td>trace (1%)</td>
<td>0.03 ± 0.01j (2.0)</td>
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<td>0.03 ± 0.01j (2.0)</td>
<td>0.55 ± 0.09e (24.9)</td>
</tr>
<tr>
<td>18:3</td>
<td>trace (1%)</td>
<td>trace (1%)</td>
</tr>
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<tr>
<td>20:4</td>
<td>trace (1%)</td>
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<td>trace (1%)</td>
</tr>
<tr>
<td>Total</td>
<td>4.71 ± 0.49f,n</td>
<td>1.28 ± 0.23n</td>
</tr>
<tr>
<td></td>
<td>2.18 ± 0.23f,q</td>
<td>1.09 ± 0.04q</td>
</tr>
</tbody>
</table>

- **a.** ug lipid/ug DNA ± SEM. Each value represents the mean from separate analyses of 4 tissue pools of at least 2 birds per pool.
- **b.** Values in parenthesis are relative percentages.
- **c-q.** Values with the same superscript are significantly different (p < 0.05).
- **c-j.** Differences between breeds.
- **k-q.** Differences between aortic sites.
<table>
<thead>
<tr>
<th></th>
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<tr>
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<td></td>
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<tr>
<td><strong>6 weeks</strong></td>
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<td></td>
</tr>
<tr>
<td>PUFA/Sat</td>
<td>52/48 (1.08)</td>
<td>67/33 (2.03)</td>
</tr>
<tr>
<td><strong>6 months</strong></td>
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<td></td>
</tr>
<tr>
<td>PUFA/Sat</td>
<td>17/83 (0.21)</td>
<td>12/88 (0.14)</td>
</tr>
</tbody>
</table>

TABLE 8: RATIOS OF UNSATURATED (UNSAT) TO SATURATED (SAT) AND POLYUNSATURATED (PUFA) TO SATURATED NON-ESTERIFIED FATTY ACIDS IN WHITE CARNEAU (WC) AND SHOW RACER (SR) AORTAS
Figure 2: Total Lipid Content as a Function of Age and Aortic Site in White Carneau (WC) and Show Racer (SR) Pigeon Aortas
Figure 3: Cholesterol (CHOL) Content as a Function of Age and Aortic
Site in White Carneau (WC) and Show Racer (SR) Pigeon
Aortas
Figure 4: Non-Esterified Fatty Acid (NEFA) Content as a Function of Age and Aortic Site in White Carneau (WC) and Show Racer (SR) Pigeon Aortas.
Figure 5: Triacylglycerol (TG) Content as a Function of Age and Aortic Site in White Carneau (WC) and Show Racer (SR) Pigeon Aortas
The diagram shows the change in ug TG / ug DNA over time, measured at different ages:

- **WC CELIAC FOCI**
- **WC THORACIC**
- **SR CELIAC FOCI**
- **SR THORACIC**

The x-axis represents the age in weeks (1 day, 6 wks, 12 wks, 6mo) and the y-axis represents ug TG / ug DNA. The data points indicate a consistent increase in ug TG / ug DNA across all age groups.
Figure 6: Cholesteryl Ester (CE) Content as a Function of Age and Aortic Site in White Carneau (WC) and Show Racer (SR) Pigeon Aortas
WC CELIAC FOCI
WC THORACIC
SR CELIAC FOCI
SR THORACIC

ug CE/ug DNA

1 day 6 wks 12 wks 6 mo

AGE
Figure 7: Phospholipid (P-Lipid) Content as a Function of Age and Aortic Site in White Carneau (WC) and Show Racer (SR) Pigeon Aortas
Figure 8: Squalene Content as a Function of Age and Aortic Site in White Carneau (WC) and Show Racer (SR) Pigeon Aortas
2. Oxidative Phosphorylation in Arterial Tissue

Aside from slight increases with age in both SR aorta sites, there was little difference in P/O ratios between WC and SR aortas in either site except for a dramatic decrease by 6 months in WC celiac foci with either \( \alpha \)-hydroxybutyrate or succinate as substrate (Table 9, page 59; Figures 9 and 10, pages 63, 65). The lower P/O ratios in WC celiac cushions were due to a lesser utilization of inorganic phosphate than in SR (Table 10, page 60).

Opposite trends in age-related changes in P/O ratios and in rates of phosphate utilization were found between the two breeds from 12 weeks to 6 months of age. Show Racer celiac foci had significantly higher P/O ratios with \( \alpha \)-hydroxybutyrate than did WC during this age period due to greater utilization of inorganic phosphate.

No significant breed differences were detected in P/O ratios in thoracic aortas for the four ages studied.

3. ATP-Dependent NADH Transhydrogenation

Fluorometric studies of energy-linked transhydrogenation revealed that aortic elementary transfer particles (ETP) from 6-week, 12-week, and 6-month old WC celiac foci carried out the reduction of NADF by NADH produced from NAD in the absence of ATP (Addition of ATP did not affect the reduction process.), while the reduction process in WC thoracic aortas, SR celiac foci, and SR thoracic aortas required ATP at all ages studied (Table 11, page 61; Figure 11, page 67). However, partial reduction was noted in 6-month old WC thoracic aortas without ATP, but it was required to complete the reaction (Figure 12, page 69).
### TABLE 9: P/O RATIOS IN WHITE CARNEAU (WC) AND SHOW RACER (SR) AORTIC MITOCHONDRIA

<table>
<thead>
<tr>
<th>Age</th>
<th>Substrate</th>
<th>WC</th>
<th>SR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>celiac foci</td>
<td>upper thoracic</td>
</tr>
<tr>
<td>1 day</td>
<td>β-OH butyrate</td>
<td>2.21 ± 0.24a (5)</td>
<td>2.48 ± 0.47 (3)</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>1.33 ± 0.07 (5)</td>
<td>1.59 ± 0.25 (3)</td>
</tr>
<tr>
<td>6 weeks</td>
<td>β-OH butyrate</td>
<td>2.60 ± 0.47 (4)</td>
<td>2.05 ± 0.13 (4)</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>1.38 ± 0.10 (3)</td>
<td>1.22 ± 0.04 (3)</td>
</tr>
<tr>
<td>12 weeks</td>
<td>β-OH butyrate</td>
<td>2.34 ± 0.13b (3)</td>
<td>2.29 ± 0.15 (3)</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>1.61 ± 0.20c (3)</td>
<td>1.31 ± 0.06 (3)</td>
</tr>
<tr>
<td>6 months</td>
<td>β-OH butyrate</td>
<td>1.04 ± 0.14b,g,i (4)</td>
<td>2.08 ± 0.25i (4)</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>0.99 ± 0.09c,h,j (4)</td>
<td>1.30 ± 0.14j (4)</td>
</tr>
</tbody>
</table>

a. P/O ratios ± SEM for the number of mitochondrial pools shown in parenthesis. Approximately equal numbers of male and female aortas were used to obtain each pool.
b-j. values with the same superscript are significantly different (p < 0.05).
b-f. age differences in each aortic site within each breed.
g,h. differences between breeds.
i,j. differences between aortic sites.
### TABLE 10: RATES OF PHOSPHOROUS AND OXYGEN UPTAKE IN WHITE CARNEAU (WC) AND SHOW RACER (SR) AORTIC MITOCHONDRIA

<table>
<thead>
<tr>
<th>Age</th>
<th>WC</th>
<th>SR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>celiac foci</td>
<td>upper thoracic</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>O</td>
</tr>
<tr>
<td></td>
<td>butyrate</td>
<td>butyrate</td>
</tr>
<tr>
<td>1 day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>1.56 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.20 ± 0.46</td>
</tr>
<tr>
<td>O</td>
<td>0.72 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.89 ± 0.05</td>
</tr>
<tr>
<td>succinate</td>
<td>1.00 ± 0.06</td>
<td>1.23 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>0.76 ± 0.03</td>
<td>0.82 ± 0.13</td>
</tr>
<tr>
<td>6 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>2.28 ± 0.45</td>
<td>1.49 ± 0.44</td>
</tr>
<tr>
<td>O</td>
<td>0.88 ± 0.04</td>
<td>0.64 ± 0.19</td>
</tr>
<tr>
<td>succinate</td>
<td>1.12 ± 0.02</td>
<td>1.13 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>0.82 ± 0.04</td>
<td>0.83 ± 0.04</td>
</tr>
<tr>
<td>12 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>1.23 ± 0.09</td>
<td>1.42 ± 0.08</td>
</tr>
<tr>
<td>O</td>
<td>0.53 ± 0.03</td>
<td>0.63 ± 0.05</td>
</tr>
<tr>
<td>succinate</td>
<td>0.85 ± 0.19</td>
<td>0.94 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>0.52 ± 0.07</td>
<td>0.69 ± 0.02</td>
</tr>
<tr>
<td>6 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>0.73 ± 0.15&lt;sup&gt;c&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.84 ± 0.20&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>O</td>
<td>0.71 ± 0.12</td>
<td>0.71 ± 0.09</td>
</tr>
<tr>
<td>succinate</td>
<td>0.76 ± 0.06&lt;sup&gt;d&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.26 ± 0.06&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.76 ± 0.07</td>
<td>0.85 ± 0.03</td>
</tr>
</tbody>
</table>

<sup>a</sup> P: micromoles Pi utilized/hr/mg mitochondrial protein ± SEM.
<sup>b</sup> O: micromoles O consumed/hr/mg mitochondrial protein ± SEM.
<sup>c-h</sup> values with the same superscript are significantly different (p < 0.05).
<sup>c,d</sup> differences between breeds.
<sup>e</sup> differences between aortic sites.
<sup>f-h</sup> age differences for 2 aortic sites within each breed.
### TABLE 11: SUMMARY OF NADH TRANSHYDROGENATION PROFILES IN WHITE CARNEAU (WC) AND SHOW RACER (SR) AORTIC ELEMENTARY TRANSFER PARTICLES

<table>
<thead>
<tr>
<th></th>
<th>WC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SR&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>celiac foci</td>
<td>celiac foci</td>
</tr>
<tr>
<td></td>
<td>thoracic aortas</td>
<td>thoracic aortas</td>
</tr>
<tr>
<td>1 day</td>
<td>Regulation&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Regulation</td>
</tr>
<tr>
<td>6 weeks</td>
<td>No Regulation&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Regulation</td>
</tr>
<tr>
<td></td>
<td>Regulation</td>
<td>Regulation</td>
</tr>
<tr>
<td>12 weeks</td>
<td>No Regulation</td>
<td>Regulation</td>
</tr>
<tr>
<td></td>
<td>Regulation</td>
<td>Regulation</td>
</tr>
<tr>
<td>6 months</td>
<td>No Regulation</td>
<td>Partial Regulation&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Partial Regulation</td>
<td>Regulation</td>
</tr>
</tbody>
</table>

<sup>a</sup> each analysis was performed twice with elementary transfer particles corresponding to 0.75 mg mitochondrial protein

<sup>b</sup> regulation implies maximal reduction of NADP<sup>+</sup> upon addition of ATP

<sup>c</sup> no regulation implies complete reduction of NADP<sup>+</sup> without ATP

<sup>d</sup> partial regulation implies ATP was required to fully complete NADP<sup>+</sup> reduction
Figure 9: P/O Ratios in Aortic Mitochondria with $\beta$-hydroxybutyrate as Substrate
Figure 10: P/O Ratios in Aortic Mitochondria with Succinate as Substrate
Figure 11: Typical Profiles of NADH Transhydrogenation in One-Day to Six-Month Old White Carneau (WC) and Show Racer (SR) Aortas

Solid line represents the profile observed for Six-Week, Twelve-Week, and Six-Month Old WC Celiac Foci.
Dotted line represents the profile observed for One-Day and Twelve-Week Old WC Thoracic Aortas, and One-Day, Twelve-Week, and Six-Month Old SR Celiac Foci and Thoracic Aortas
Figure 12: Typical Profile of NADH Transhydrogenation in Six-Month White Carneau (WC) Upper Thoracic Aortas
DISCUSSION

Accumulation of Various Lipid Classes
In Early Spontaneous Atherogenesis

Of the individual lipid classes which accumulate at the celiac foci, squalene is present in the highest proportion as early as twelve weeks of age in both breeds (Table 2, page 37). The presence of squalene has been reported in normal and diseased human aortas (Brooks et al., 1966; St. Clair et al., 1968a), in normal rabbit aortas (Stefanovich and Kajiyama, 1970), and in diseased pigeon aortas (Nicolosi et al., 1972; St. Clair et al., 1968a). Moreover, squalene accumulation has been correlated with the severity of atherosclerosis since it is reported to be irritating to the arterial intima (St. Clair et al., 1968a). Isotopic experiments using either acetate or mevalonate in the pigeon (St. Clair et al., 1968a), rat (Daly, 1971), and rabbit aorta (Avignon et al., 1971) have demonstrated up to 60% incorporation of labeled precursor into the squalene fraction. These results correlate with the findings of Cramer and Smith (1976) who suggested that squalene originates from de novo synthesis in the embryo since it is not found in yolk lipids. Accumulation of squalene was significantly greater in WC celiac cushions than in the SR only at twelve weeks of age, occurring at the same time when lower oxygen tension was first detected in subendothelial regions of the WC celiac cushions (Farber et al., 1978). Inadequate oxygen for cyclization of squalene to cholesterol by squalene monooxygenase may account for this accumulation (Lazzarini-Robertson, 1970).
1968; Lehninger, 1975). However, it is difficult to explain the lack of significant differences in squalene concentration between WC and SR celiac cushions at six months of age since low oxygen availability was demonstrated in the muscular foci of only WC at this age. Also, these results at six months of age contradict those of Nicolosi et al. (1972) who demonstrated greater quantities of squalene in WC celiac cushions than in SR cushions. No explanation for this discrepancy is apparent at present.

By six months of age, there was a significant difference in phospholipid content between WC and SR celiac foci (WC > SR). As reported by Nicolosi et al. (1972) in three-to six-month old pigeons and by St. Clair et al. (1974) in older pigeons, phospholipids constituted a large percentage of total lipids present in both breeds. Phospholipid accumulation is accelerated in the early atherosclerotic artery in several types of experimental animals and humans (Smith, 1965; Insull and Bartsch, 1966; Portman, 1970). Kalra and Brodie (1974) found a decrease in glycerol phosphate oxidation in four-month old WC aortas as compared to SR, this would increase the availability of glycerol phosphate for synthesis of phospholipids as well as triacylglycerols in the arterial wall (Portman et al., 1967) via the Kennedy pathway by six months of age. As pointed out by St. Clair (1976), however, it is not known to what extent phospholipid accumulation participates in the pathogenesis of the atherosclerotic lesion.

Triacylglycerols (TG) comprise a major portion of the neutral lipid of the normal arterial wall (St. Clair, 1976). Previous studies have indicated that TG concentration is increased as much as two-to
three-fold during atherogenesis (Howard, 1972; St. Clair et al., 1969). Unlike the patterns observed in accumulation of other lipid classes from one day to six months of age, a linear increase in TG was evident in WC celiac foci during this age span (Figure 5, page 51), with a two-fold difference in amounts between breeds at six months of age. The mechanism(s) responsible for this continual increase have yet to be elucidated. It has been suggested by Newman and co-workers (1961) that the bulk of fatty acids of arterial TGs are derived through local synthesis and are subsequently esterified to glycerol phosphate. Lack of glycerol phosphate oxidation in four-month old WC thoracic aortas compared to SR (Kalra and Brodie, 1974) and the continual availability of non-esterified fatty acids in WC celiac foci (Figure 4, page 49) are consistent with this hypothesis. Furthermore, it has been suggested by Kalra and Brodie (1974) that the lack of control of NADH transhydrogenation (which regulates the NADPH/NADP⁺ ratio) in arterial smooth muscle cells may be instrumental in driving non-esterified fatty acid and TG synthesis in the WC (Table 11, page 61). However, decreased oxidation of glycerol-3-phosphate was not detected until four months of age so substrate for TG synthesis may not be available until this age.

As an alternative to this hypothesis, Vost (1972) has proposed that chylomicron-bound TG may enter the arterial wall without first being hydrolyzed at the endothelial surface. Perhaps there is some de-endothelialization of the blood vessel wall, beginning at six weeks of age at the WC celiac artery bifurcation (Lewis and Kottke, 1977), which could allow passage of low and very low density lipoproteins, and TG-enriched chylomicrons into the
aortic wall to become subsequently trapped by accumulating glycosaminoglycans (GAGs) (Curwen and Smith, 1977). Complexes such as these may prevent diffusion of oxygen into the middle layers of the arterial wall. Or, they may enhance uptake of LDL-cholesterol by underlying smooth muscle cells.

Increased triacylglycerol accumulation seen by six months of age in WC celiac cushions (Tables 2 and 3, pages 37 and 38) may also result from a lack of sufficient lysosomal lipolytic activity (St. Clair, 1976). Under normal circumstances, arterial TGs can serve as a source of energy for the arterial wall by providing fatty acids for oxidation by the TCA cycle, or they can provide the cell with a source of fatty acids for esterification to other lipids, e.g. cholesterol. However, a large pool of non-esterified fatty acids are already present by six months of age (Table 2, page 37), which may be due to slower TCA cycle activity (Zemplenyi et al., 1975), lack of NADH transhydrogenation control (Table 11, page 61), or exogenous infiltration (Dayton and Hashimoto, 1968; Subbiah et al., 1974) occurring as early as six weeks of age. Perhaps excessive accumulation of non-esterified fatty acids inhibit lysosomal lipase activity, thereby diminishing TG lipolysis in aortic smooth muscle cells. Consequently, control of TG lipase activity by fatty acids may resemble the control mechanism of hormone-sensitive (ACTH, epinephrine) lipase activity in adipocytes (Small, 1977). In any event, six-month old WC celiac cushions may have a lesser capacity to catabolize TGs than SR cushions where there may be a greater capacity to metabolize fat at this age.
Less lysosomal lipolytic (or hydrolytic) activity may be fundamental to the pathogenesis of atherosclerosis (Small, 1977). In many lysosomal storage diseases (e.g. gangliosidosis) where appreciable decreases in lipid lipase or hydrolase activities as well as lipid accretion (O'Brien et al., 1971) have been reported, acid mucopolysaccharides also accumulate concomitantly (O'Brien et al., 1971). To explain excessive accumulation of TG, P-lipids, and cholesteryl esters as well as GAG accretion at six months of age, a possible defect in lysosomal activity can be proposed in the WC. According to this hypothesis, atherosclerosis may be a type of lysosomal storage disease.

One of the earliest-appearing lipid class differences between celiac sites was non-esterified fatty acid accumulation in the WC (Table 2, page 37). Such accumulation during early atherogenesis is consistent with other studies (St. Clair, 1976). Several hypotheses may explain this pattern. By six weeks of age, WC celiac mitochondria lack control of NADH transhydrogenation by ATP (Table 11, page 61) which can lead to higher NADPH/NADP⁺ ratios in the cell (Kalra and Brodie, 1973; 1974). This lack of regulation has been suggested to play an important role in fatty acid accumulation by increasing biosynthesis in the aorta (Kalra and Brodie, 1974; Whereat, 1970). Superimposed upon this lesser capacity of WC celiac mitochondria to regulate NADH transhydrogenation at six weeks is a shift in energy production from the TCA cycle to glycolysis (Zemplenyi et al., 1975; Zemplenyi and Rosenstein, 1975). Lower TCA cycle activity in WC aortas beginning at five to eight weeks of age also appears consistent with the hypothesis of increased NEFA production since lower TCA cycle activity would retard
fatty acid oxidation and favor synthesis by making more citrate (and acetate) available. Pathobiologic effects of NEFA accumulation include stimulation of (uncoupled) mitochondrial ATPase activity (Hajjar and Smith, 1978; Wojtczak, 1976) and structural damage to the mitochondrion to cause eventual uncoupled respiratory-chain phosphorylation (Chan and Higgins, 1978) by six months of age (Table 9, page 59). This has been stated to occur, particularly if these NEFA are palmitate, stearate, or oleate, by allowing the inner mitochondrial membrane to become permeable to protons (Wojtczak, 1976). In addition, excessive NEFA accumulation in WC celiac foci could lead to subsequent accumulation of esterified lipids, such as cholesteryl esters, phospholipids, and triacylglycerols at later ages. However, the key problem appears to be identification of the factor(s) which cause this excess of non-esterified fatty acids in six week WC celiac cushions. Perhaps smooth muscle cells in WC have an inability to convert their long chain fatty acids to acyl CoA thioesters to serve as respiratory substrates for oxidation.

Smith et al. (1965) reported that more than 45% of the total fatty acids in WC aortas were of the saturated variety (myristic, palmitic, and stearic acids), and this investigation showed that there was a rapid increase in the percentage of saturated NEFA and a decrease in (poly) unsaturated NEFA in WC celiac cushions from six weeks to six months of age (Table 8, page 43). Saturated NEFA (palmitate and stearate) were also shown to be the predominant fatty acids esterified to cholesterol at six weeks and six months of age in WC celiac cushions (Hojnacki et al., 1977).
The increase in saturated fatty acids and decrease in unsaturated fatty acids at branched portions of WC aortas by six months of development may be attributed to lower levels of oxygen found in these foci at twelve weeks and six months of age (Farber et al., 1978) since oxygen is needed for desaturation (Lehninger, 1975). As a result, reduced levels of linoleic acid, a precursor for prostaglandin (PG) E₁, E₂ and F₂α production (Hwang et al., 1975), may provide favorable conditions for cholesteryl ester accumulation since lack of these PGs may permit uncontrollable synthesis and/or insufficient stimulation of cholesteryl ester hydrolysis (Berberian et al., 1976).

It is also noteworthy that the lack of linoleate in WC celiac NEFA pools by six months of age suggests that there may be a lack of this essential fatty acid (EFA) available for production of phospholipids. Although the validity of this idea has yet to be confirmed by analyses of P-Lipid fatty acids, tightly coupled respiratory-chain phosphorylation could be affected since linoleate and arachidonate are necessary in mitochondrial phospholipids for efficient oxidative energy production (Racker, 1970). Smith et al. (1966) found that aging intimal cells cultured from WC aortas were deficient in EFAs when compared with similar cells cultured from SR aortas, and that they lacked histological-demonstrable mitochondrial ATPase. Also, Ito and Johnson (1964) found low respiratory control quotients in EFA deficient rats. Correlation of these reports suggests a mitochondrial coupling defect in WC aortic intimal cells related to lower levels of EFAs.

Aortic tissue can synthesize saturated fatty acids (St. Clair et al., 1968b), elongate fatty acid chains, and desaturate in certain
positions (St. Clair, 1976). However, this organ cannot produce linoleate (Velican, 1974), so linoleate probably arises from plasma lipoproteins (containing cholesteryl linoleate) perfusing through the arterial wall (Smith et al., 1967; Velican, 1974).

It is unlikely that the saturated (myristic, palmitic and stearic) and monoenoic (myristoleic and oleic) acids accumulating in WC celiac cushions originate from only one source since Subbiah et al. (1974) found these fatty acids to be present in the plasma; and St. Clair et al. (1968b) demonstrated that pigeon aortas synthesize myristic, palmitic, stearic, and oleic acids in situ. Consequently, the origin and/or mechanism for accumulation of these NEFA remains to be elucidated.

There is substantial evidence (Insull and Bartsch, 1966; Portman et al., 1967) that intima of human aortas accumulates large quantities of cholesterol before cholesteryl esters increase. Such a pattern is consistent with the trend observed in this study (Figures 3 and 6, page 47 and 53) and renders support to the concept that lipid accretion in pigeons parallels closely that pattern seen for humans (Portman, 1970; Clarkson et al., 1973).

If pigeon arterial smooth muscle cells accumulate excess cholesterol by twelve weeks of age this may increase the likelihood of its incorporation into the plasma membrane (Jackson and Gotto, 1976), thereby decreasing its fluidity. This can seriously hamper metabolic activities (Papahadjopoulos, 1974). The cell can subsequently compensate by increasing the esterification of cholesterol (Jackson and Gotto, 1976) via fatty acyl CoA cholesteryl acyl transferase (ACAT) (St. Clair, 1976) using available NEFA. It has been further proposed
(Papahadjopoulos, 1974) that accumulation of intracellular cholesteryl esters can affect the subsequent release of cholesterol from the cell by inhibiting normal secretory processes. In any event a five-fold difference in both cholesterol and cholesteryl ester content between WC and SR celiac cushions was noted by six months of age (Table 2, page 37). Less esterified cholesterol in the SR may permit more efficient release of free cholesterol from the smooth muscle cells.

The presence of large amounts of steryl esters in celiac cushions has been demonstrated in several animal species including humans, and their accumulation correlates with the severity of the disease (Day and Wahlqvist, 1970; Dayton and Hashimoto, 1968; Lofland et al., 1965). The pattern of cholesterol/cholesteryl ester accretion seen in WC pigeon celiac cushions and thoracic aortas (Figures 3 and 6, pages 47, 53) is very similar to that described by Hojnacki et al. (1977). Based on the study by Hojnacki and co-workers (1977) as well as this investigation, the 'critical' period of spontaneous sterol and steryl ester accumulation at WC lesion sites occurs between twelve weeks and six months of age (Figures 3 and 6, pages 47, 53). Subbiah et al. (1976) identified the critical period to be around one year of age, but these investigators did not examine focal aortic sites predisposed to lesion formation.

Data presented in Figures 3, 4, and 6 (pages 47, 49, and 53) indicate a distinct sequence of appearance of cholesterol, NEFA, and cholesteryl esters in WC and SR celiac foci. Differences in ACAT or lysosomal/cytosolic cholesteryl ester hydrolase activity may account for variations in sterol and steryl ester content during the critical period of accumulation when breed differences were
detected. Experiments by Bonner et al. (1972) are supportive of this concept since they demonstrated that species resistant to atherosclerosis had higher cholesteryl ester hydrolytic activity than those susceptible to the disease during an age period corresponding to early atherosclerosis. A similar conclusion was obtained by comparing cholesteryl ester synthetase and hydrolase activities in aortas from WC pigeons with those in SR aortas (Kritchevsky and Kothari, 1973). In these studies, hydrolytic activity exceeded synthesis in the resistant breed (SR), whereas synthetic activity exceeded hydrolysis in the susceptible breed (WC). Accelerated synthesis may be explained by the substantial availability of substrate (cholesterol, free fatty acids) to produce cholesteryl esters by six months of age. Recently, Hojnacki et al. (1977) demonstrated that the major cholesteryl esters accumulating in WC celiac cushions by six months of age were cholesteryl palmitate and cholesteryl stearate, yet Subbiah and Dicke (1976) have shown that cholesteryl ester hydrolase activity in the WC and SR aortas is much higher when cholesteryl linoleate and cholesteryl oleate are available. Consequently, the accumulation of cholesteryl palmitate and cholesteryl oleate may be due to less rapid hydrolysis. It is impossible to deduce the precise contribution of endogenous cholesterol esterification to the accretion of cholesteryl esters during this period of atherogenesis (twelve weeks to six months of age), although cholesterol esterification has been well documented to be an active process within the arterial wall. Studies of pigeon lipid biosynthesis in aortic tissue (Lofland et al., 1960; St. Clair et al., 1968b) have demonstrated that the synthesis of saturated fatty acids, viz., palmitate and stearate, followed by their rapid esterification to the pre-existing pool of
cholesterol, is an active, dynamic process. Aside from the investigation of Hojnacki et al. (1977) which was previously described, Dayton and Hashimoto (1968), as well as Kothari et al. (1973), have suggested that up to 50% of arterial cholesteryl esters in the rabbit are synthesized in situ. (The remaining cholesteryl esters are derived from infiltrating serum lipoproteins.)

However, stimulation of cholesterol esterification may be secondary to the binding of low density lipoproteins (LDL) to specific binding sites on the plasma membrane of aortic smooth muscle cells (St. Clair, 1976). It is possible that the number of these binding sites are genetically controlled, as appears to be the case for skin fibroblasts (Brown and Goldstein, 1976). Or, these lipoproteins may stimulate pinocytosis, resulting in an increase in the already extensive system of plasmalemma vesicles within the smooth muscle cell (St. Clair, 1976). This could result in an uptake of cholesteryl esters from lipoproteins. Although lipoproteins have been demonstrated in the arterial wall (St. Clair, 1976), there is no clear indication whether low or high density lipoprotein-bound cholesteryl esters represent the major source from which "intracellular" cholesterol/cholesteryl esters originate during early spontaneous pigeon atherogenesis (Jensen et al., 1978). Nonetheless, conflicting studies have indicated that the sterol portion of cholesteryl esters which accumulate in atherosclerotic arteries is derived from the blood and is not synthesized to a large extent in the blood vessel wall (Lofland and Clarkson, 1970; St. Clair, 1976).

It has been pointed out by Goldstein and Brown (1975) that there are increases in the incorporation of cholesterol and cholesteryl esters
from LDLs (Rothblat et al., 1967; Small, 1977) into smooth muscle cells when intracellular de novo biosynthesis of cholesterol and its subsequent esterification decreases. In this case, cells compensate by producing more cell-surface LDL-cholesterol/cholesteryl esters receptors (Bierman et al., 1974; Goldstein and Brown, 1975). Consequently, more LDL from the plasma bind to the cell surface receptors and are then incorporated into the cell to increase the intracellular pools of free and esterified cholesterol. Cholesterol derived from the plasma then suppresses further cholesterol biosynthesis (Bierman et al., 1974; Lehninger, 1975).

Role of Bioenergetic Metabolism in Early Spontaneous Atherogenesis

Although it has been purported that accumulation of extracellular matrix components (viz., GAGs) are responsible for trapping serum lipoproteins (Curwen and Smith, 1977; Iverius, 1972), accumulation of proteoglycans may contribute very little to spontaneous lipid accumulation in WC celiac cushions prior to twelve weeks of age since it is characterized by high concentrations of NEFA. Most NEFA are not transported by lipoproteins but rather by albumin which does not bind appreciably to GAGs (Iverius, 1972). Consequently, alternative explanations are needed to explain NEFA accretion, the earliest appearing lipid abnormality.

Beginning at one day of age, Cramer and Smith (1976) found less utilization of yolk lipids in the WC, which may be due to decreased oxidation and perhaps related to the rapid accumulation of NEFA between one day and six weeks of age. Superimposed upon this posited lesser capacity to metabolize lipid, alterations in energy metabolism in WC
foci were detected at six weeks of age (the earliest age studied).
Lack of NADH transhydrogenation control by ATP was noted in these foci compared to the SR at this age and subsequent ages (Table 11, page 61). Because it was virtually impossible to isolate sufficient aortic mitochondria, transhydrogenation was not studied in one day celiac foci, so it is possible that this lack of control may be present much earlier and be related to NEFA accretion at one day of age. In any event, the lack of ATP control of NADP⁺ reduction via NADH in WC celiac mitochondria at six weeks and subsequent ages could lead to an increase in the ratio of NADPH/NADP⁺ thereby promoting fatty acid synthesis as explained previously.

It is also noteworthy that the lack of control of NADH transhydrogenation by ATP suggests an aberration in the regulatory mechanism for the production of redox energy (in the form of NADPH) at WC lesion sites in which a high NADPH/NADP⁺ ratio can promote lipid synthesis (Kalra and Brodie, 1974; Lehninger, 1975). Larger pools of pre-existing NEFA in one-day WC celiac cushions than in SR may be binding with adenine or guanine nucleotides for the NADP⁺ binding site on transhydrogenase so that ATP can not exert its regulatory action (Rystrom, 1972; Wojtczak, 1976). Consequently, a defect in the control mechanism for the production of NADPH is indicated. Aside from the alterations noted in the control of transhydrogenation in WC celiac foci, it is possible that the mechanism for the actual transfer of energy to drive respiratory-chain phosphorylation may be atypical since the nature and location of the electron transfer catalyst (ETC) (high energy intermediate) may be different. The insensitivity of NADH transhydrogenase to dicumarol in the WC is consistent with this hypothesis since the ETC
may be deeply buried in the inner mitochondrial membrane or protected by lipid annuli so that phenol derivatives have a minimal effect. Furthermore, the NADH transhydrogenation profile from WC celiac ETPs demonstrated not only loss of ATP regulation, but also an inability for regulation of transhydrogenase by other high energy compounds from the mitochondria such as GTP which possess different phosphate group potentials than ATP (Danielson and Ernster, 1963). Since tightly coupled oxidative phosphorylation and increased coupled mitochondrial $F_1$ ATPase were demonstrated at this age in WC celiac mitochondria, it does not seem probable that lack of control can be attributable to the lack of ATP. Moreover, the cause of this inability to use ATP may be genetic in nature (Kalra and Brodie, 1974). The enzyme may be defective in WC embryos; perhaps the enzyme has a different conformation such that ATP or other high energy compounds can not bind to regulate activity. It would be interesting to test this hypothesis in mitochondria derived from embryonic aortas or from other embryonic tissues such as liver.

During the period from six weeks to twelve weeks of development, there was significant lipid accumulation in both breeds at the celiac foci, but not in thoracic regions. Possessing a greater capacity to resist lipid accumulation, cells derived from the upper thoracic region may be more mature than those derived from the celiac region during this period of development (Santerre et al., 1972). As maturation continues, cells from the SR celiac foci soon gain a similar capacity, having a more tightly coupled phosphorylation system (Figure 9, page 63) to facilitate metabolism (Klingenberg and Bode, 1965; Spector, 1971) or for excretion of lipid (Rothblat et al., 1967).
It is noteworthy that the SR resists those metabolic aberrations seen in the WC celiac foci during early atherogenesis.

If de-endothelialization of the WC arterial wall at the celiac foci occurs beginning at six weeks of age (Lewis and Kottke, 1977), a favorable situation would be provided for cholesterol to accumulate via the lipoprotein infiltration process previously discussed. Lipoproteins trapped by accumulating GAGs during this age period (Curwen and Smith, 1977) may be forming oxygen diffusion barriers in the extracellular matrix of the WC blood vessel wall. This can cause smooth muscle cells to become hypoxic (Filipovic and Buddecke, 1971; Lazzarini-Robertson, 1968); and, if this situation worsens to the point where there is substantial blockage of oxygen diffusion, eventual impairment of mitochondrial function can ensue (Whereat, 1967). Particularly, a loss of energy production would result by uncoupling oxidative phosphorylation from electron transport (Zemplenyi, 1968). Low oxygen tension in subendothelial regions of the WC celiac cushions was observed at twelve weeks and six months of age (Farber et al., 1978). It was at this later age, after the initial observation of diminished oxygen availability to the intimal regions, that the WC celiac mitochondria exhibited lower P/O ratios than the SR, suggesting a loosely coupled system and an inability to metabolize the accumulating lipid during this age period as a result of this loss in energy production (Klingenberg and Bode, 1965; Spector, 1971).

Other biochemical derangements have been associated with hypoxia, specifically concerning bioenergetics. Increased synthesis of lipids (triacylglycerols, phospholipids, NEFA) due to increased levels of NADPH/NADP⁺ (Filipovic and Buddecke, 1971) or lowered TCA
cycle activity (Kinnula and Hassinen, 1978) have been identified during periods of decreasing oxygen availability to cells. Hypoxia during this age period may also promote increased lipid uptake by causing increased endothelial permeability (Lazzarini-Robertson, 1968), by creating energy deficits by altering the ATP/ADP ratio (Filipovic and Rutemoller, 1976) through uncoupled respiratory-chain phosphorylation or inhibition of the adenine nucleotide translocase system (Christiansen and Davis, 1978), or by decreasing lipid excretion (Gordon et al., 1977; Rothblat et al., 1967). Contrary to this metabolic situation, the tightly coupled oxidative phosphorylation found in SR aortas (Table 9, page 59) may enable vascular smooth muscle cells to maintain lower NADH/NAD+ ratios and higher levels of oxidatively formed ATP, giving them a greater ability to metabolize or exclude lipids.

Experiments performed to assess the capacity for coupled mitochondrial phosphorylation in WC vascular smooth muscle cells during early atherogenesis revealed similar P/O ratios in SR from one day to twelve weeks of age, and this correlates with the findings of Kalra and Brodie (1974) who used twelve-week old WC and SR thoracic aortas. It was not until six months of age that the initial loss of energy production was observed in WC celiac foci compared to the SR (Table 9, page 59). These results closely parallel the studies of Santerre et al. (1974) who reported a loss of respiratory control with succinate in WC celiac cushions at six months of age.

Although decreased coupled, respiratory-chain phosphorylation was demonstrated with succinate or \( \beta \)-hydroxybutyrate as the electron donor (Figures 9 and 10, pages 63, 65) a larger decrease was observed with \( \beta \)-hydroxybutyrate. Since the electrons derived from \( \beta \)-hy-
d Rox ybutyrate enter the respiratory chain prior to coupling site I in the electron transport system (Lehninger, 1964), and there was a lack of control of NADH transhydrogenase (which normally transduces available redox energy for utilization via electron transport system), it would appear that the site of ATP generation from the respiratory chain is defective in the WC beginning at complex I of the mitochondrial oxidative phosphorylating system (Hatefi and Stiggall, 1976) (c.f. below.)

Aortic mitochondria, damaged either by hypoxia, pre-existing lipid (free fatty acids and cholesterol), or both, accumulate more lipid and contribute to atherogenesis (Murray et al., 1968; Smith et al., 1966).

Ultrastructurally abnormal mitochondria associated with lipid vacuole formation have been observed in six-month old WC celiac cushions (unpublished observations.) A damaged mitochondrial membrane will cause
lower energy production at any one of the complexes of the mito-
chondrial oxidative phosphorylation system (Hatefi and Stiggall, 1976).
Low P/O ratios observed in six-month old WC celiac mitochondria are
attributable to appreciable decreases in the rate of phosphate
utilization (Table 10, page 60) suggesting an impairment in the
mechanism for respiratory-chain phosphorylation. Decreased coupled
mitochondrial $F_1$ ATPase activity in six-month old WC aortas, suggest-
ing a loss of oxidative phosphorylation (Hajjar and Smith, 1978),
appears consistent with these findings. Alternatively, the lowered
P/O ratios may be explained by the inhibition of the adenine translocase
reaction, the rate-limiting step of oxidative phosphorylation, which
can induce a lower phosphorylation potential (Wojtczak, 1976).
Accumulating fatty acids, particularly the larger deposits of saturated
fatty acids (Table 8, page 43), have the potential to competitively
bind to ADP-specific receptor sites of the translocase and thereby
prevent sufficient mobilization of ADP to the site of phosphorylation
for subsequent utilization by the $F_1$ coupling factor protein along
the inner mitochondrial membrane. These effects on mitochondrial
energy-coupling reactions (i.e., lowering of P/O ratios and inhibition
of coupled mitochondrial $F_1$ ATPase activity) have been well established
(Lerner et al., 1972; Pande and Blanchaer, 1971; Shrago et al., 1974).

In previous studies, Santerre et al. (1974) also proposed a
defect in the coupling mechanism associated with $F_1$ ATPase since there
was an inability of six month old WC celiac tissue to respond to added
ATP during state 3 respiration. If a normal coupling system were
simply uncoupled, a much higher state 4 respiration rate would be
expected than in SR. This was not the case. Owing to this defect
in the phosphorylating coupling system, lipid accretion occurs, thus accelerating the pathogenesis of atherosclerosis.

Consequently, a sequential metabolic pattern has emerged such that initial non-esterified fatty acid accumulation occurs prior to uncoupled oxidative phosphorylation. Beginning at six weeks of age, increased synthesis of non-esterified fatty acids is suggested owing to: (1) the lack of control of ATP of NADH transhydrogenation thereby increasing the potential for a higher NADPH/NADP⁺ ratio in the cell; and (2) the decreased TCA cycle activity and subsequent accumulation of citrate and acetate (Zemplenyi and Rosenstein, 1975). Increased synthesis may be 'over-riding' the ability of the WC smooth muscle cells to adequately catabolize these fatty acids at this age; and, occurring subsequent to less TCA cycle activity, uncoupled respiratory-chain phosphorylation and decreased mitochondrial ATPase activity in WC celiac mitochondria could be responsible for lesser formation of fatty acyl-CoA derivatives causing diminished "fatty acid activation" for oxidation (Lehninger, 1975; Wojtczak, 1976). Perhaps the high levels of NEFA in six-week to six-month WC celiac foci promote the accretion process of esterified lipids by six months of age as substrates (glycerol phosphate, cholesterol) become available at twelve weeks of age (Table 2, page 37; Kalra and Brodie, 1974). Continual increased fatty acid synthesis and concomitant decreased oxidation may account for the net increase in total NEFA, even at an age of development when esterified lipids are accumulating.

In any event, the decline in energy transducing capability of WC celiac cushions with maturation may reflect diminishing ability of this organ to eliminate intracellular materials associated with the
pathogenesis of atherosclerosis. In the SR aortas, the increasing capacity for ATP production (Figures 9 and 10, pages 63 and 65) and lack of hypoxic stress (Farber et al., 1978) from one day to six months of age, suggests a metabolically favorable situation for withstanding lipid accumulation and correlates well with the observed higher RCR and resistance to atherogenesis (Santerre et al., 1972; 1974).
CONCLUSIONS

It is obvious from this investigation that a number of metabolic parameters are altered during pre- and early atherogenesis. Prior to this study, the chronological relationship between oxidative energy metabolism and lipid accumulation remained unclear. However, based on these results, loss of oxidative energy production in six-month old WC celiac cushions does not appear to be the primary factor involved in initiating lipid accretion, even though accentuation of lipid deposition subsequent to six months of age may be the result of decreasing ATP production. However, a defect in mitochondrial NADH transhydrogenation was detected as early as six weeks of age and may contribute to atherogenesis since this process is believed to control fatty acid and triacylglycerol synthesis. Also, high concentrations of NEFA, one of the earliest-appearing abnormalities, may be esterified to produce accumulations of other lipids (CE, TG, P-lipids) at older ages. Decreased oxidative catabolism of fatty acids reflected by lower TCA cycle activity (build-up of acetate and citrate), and a deficiency in the glycerol phosphate shuttle in WC aortas (causing glycerol phosphate accumulation) have also been suggested as possible causes for lipid accretion in the arterial wall.

However, it is difficult at this time to identify a single factor which, by itself, would satisfactorily explain the observed increased deposition of NEFA in WC celiac cushions from one day to six months of age, or the increased accumulation of other lipid classes by six months of age. Aside from early alterations in mitochondrial metabolism in the
WC, the lesser capacity to utilize yolk lipids and the accumulation of proteoglycans have been proposed to serve as contributing factors to lipid accretion. It is quite likely that a combination or sequence of metabolic aberrations in WC celiac foci are involved in the pathogenesis of atherosclerosis.

The following diagram is presented to summarize metabolic differences between WC celiac sites and corresponding SR foci during early spontaneous atherogenesis:
<table>
<thead>
<tr>
<th>Whole Embryo</th>
<th>1 Day</th>
<th>6 Weeks</th>
<th>12 Weeks</th>
<th>6 Months</th>
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<tr>
<td>Lesser Utilization of Yolk Lipids: (CE, TG, CHOL, P-Lipid)</td>
<td></td>
<td>Lack of NADH</td>
<td>Transhydrogenation Control by ATP</td>
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<tr>
<td>(Cramer and Smith, 1976)</td>
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<td>Decreased TCA cycle activity and Increased Glycolysis</td>
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<td>Uncoupled Oxidative Phosphorylation Ultrastructurally Abnormal Mitochondria</td>
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<td></td>
<td></td>
<td>Increased NEFA</td>
<td>Decreased Glycerol Phosphate Oxidation (Kalra and Brodie, 1974)</td>
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<td></td>
<td></td>
<td></td>
<td>Increased CHOL</td>
<td>Increased CE, TG, P-Lipid - Lipid Vacuoles</td>
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<td></td>
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<td>Increased F₁ ATPase (Hajjar and Smith, 1978)</td>
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<td></td>
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<td></td>
<td>Increased GAG (Ch-4-S, Ch-6-S)</td>
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<td></td>
<td></td>
<td></td>
<td>(Curwen and Smith, 1977)</td>
<td>Hypoxia (Farber et al., 1978)</td>
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<td></td>
<td>Variant HS (Curwen and Smith, 1977)</td>
<td>Ultrastructural Dilation of Granular Endoplasmic Reticulum (ER)</td>
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Consequently, the next steps are to design experiments to determine: (1) the cause(s) of NEFA accumulation in WC celiac foci (decreased $\beta$-oxidation, increased synthesis, or both); (2) the major mechanism(s) responsible for the subsequent accumulation of cholesterol/cholesteryl esters and triacylglycerols (increased formation from accumulating substrates, decreased catabolism or removal, etc.); and (3) the profile of mitochondrial NADH transhydrogenation in celiac foci at ages prior to large increases in NEFA accretion (< six weeks), including the actual determination of the NADPH/NADP$^+$ ratio at one day and six weeks of age.


BIOGRAPHICAL DATA

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Collegiate Institutions Attended

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Honors or Awards
- B.A. degree awarded with Honorable Distinction in Biological Chemistry, 1974
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- Alpha Chi
- Phi Sigma Phi
- Phi Sigma
- Sigma Xi

Publications


Positions Held

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<td>Research Assistantship</td>
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<td>Department of Animal Sciences - UNH</td>
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