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CHOLESTERYL ESTERS IN AORTAS AND AORTA CELL CULTURES FROM ATHEROSCLEROSIS SUSCEPTIBLE AND ATHEROSCLEROSIS RESISTANT PIGEONS

JEROME LOUIS HOJNACKI

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by

JEROME LOUIS HOJNACKI

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This thesis has been examined and approved.

Samuel C. Smith

Thesis director, Samuel C. Smith,
Professor of Biochemistry
and Animal Sciences

Frank K. Hoornbeek

Frank K. Hoornbeek, Associate
Professor of Zoology and Genetics

John J. Sasner, Jr.

John J. Sasner, Jr., Associate
Professor of Zoology

Elizabeth C. Smith

Elizabeth C. Smith, Lecturer
in Animal Sciences

R. G. Strout

Richard G. Strout, Professor
of Animal Sciences

Edward K. Tillinghast

Edward K. Tillinghast,
Associate Professor of Zoology

9/9/75

Date

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ABSTRACT

CHOLESTERYL ESTERS IN AORTAS AND AORTA CELL CULTURES FROM ATHEROSCLEROSIS-SUSCEPTIBLE AND ATHEROSCLEROSIS-RESISTANT PIGEONS

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In an effort to facilitate analyses of cholesteryl esters from pigeon aorta tissue and aorta cell cultures, a thin-layer chromatographic procedure was developed which permitted the rapid separation of cholesteryl esters from other lipids. Through the sequential use of two solvent systems, six lipid classes were separated on one thin-layer chromatogram. In addition to saving substantial amounts of time, this procedure permitted the in situ fluorometric or densitometric analysis of the lipid classes and prevented losses of microgram quantities of lipid which often result from scraping, elution, and rechromatographing for separation by conventional procedures.

With this procedure followed by gas-liquid chromatography, the amount and fatty acid composition of cholesteryl esters from upper thoracic aortas and from muscular foci at the celiac bifurcation of the aorta in 1-day-old, 6-week-old, and 6-month-old White Carneau and Show Racer pigeons were determined. Although there were no significant changes in the cholesteryl ester composition of the upper thoracic aortas, linear increases in total

cholesteryl ester as a function of age, as well as changes in proportions of various fatty acids esterified to cholesterol, were observed in celiac foci of both breeds. Accumulation of saturated cholesteryl esters accounted for the increase in total cholesteryl esters during the first 6 weeks while accumulation of monounsaturated and polyunsaturated cholesteryl esters was responsible for the increase during the 6-week to 6-month period.

Since intracellular and extracellular cholesteryl esters may have different origins and since the separation of cellular lipid from extracellular lipid in intact aorta tissue is equivocal, White Carneau and Show Racer aorta cell cultures were used to study the accumulation of cellular and extracellular cholesteryl esters. Cultures were fractionated into cell, extracellular matrix, and culture medium fractions. For both breeds the pattern of cholesteryl ester fatty acids in the matrix fraction resembled that of the culture medium while the cell fraction was distinctly different from both the matrix and culture medium fractions. This suggested that aorta cells in culture may form cholesteryl esters by in situ esterification while the extracellular matrix may bind and concentrate esters from the culture medium. The significantly higher esterified to free cholesterol ratios in White Carneau cell and matrix fractions when compared to similar fractions in Show Racer cultures, indicated that White Carneau cultures accumulated relatively more cholesterol in the esterified form than in the free form.

Correlation of the in vivo and in vitro analyses suggested that, initially, aorta tissue at the celiac bifurcation resembled

aorta smooth muscle cells in culture in the synthesis of predominantly saturated cholesteryl esters. The increase in cholesteryl oleate and cholesteryl linoleate in celiac foci after 6 weeks of age, however, probably reflected a transition in synthesis from saturated to monounsaturated cholesteryl esters and the increased infiltration and deposition of plasma lipoproteins rich in polyunsaturated cholesteryl esters.

In an effort to determine the process(es) by which aorta cells in primary culture accumulate cholesteryl esters, White Carneau and Show Racer aorta cultures were incubated with culture medium containing cholesterol - 4 - ^{14}C, and aliquots of these cultures were harvested at 12 hr intervals for a 48 hr period. Although rates of incorporation of labeled cholesterol and accumulation of labeled cholesteryl ester were similar for cells from both breeds, a linear increase in the mass of cholesteryl esters was observed only in White Carneau cells throughout the culture period. After 12 hr in culture the extracellular matrix of Show Racer cells accumulated more newly synthesized cholesteryl - 4 - ^{14}C ester than did the matrix of White Carneau cells. It is suggested that accumulation of cholesteryl esters in White Carneau cells may result from slower excretion and/or slower catabolism relative to Show Racer cells.

INTRODUCTION

It was reported in 1974 that diseases of the cardiovascular system were responsible for 54 percent of all deaths in the United States, and that 84 percent of this cardiovascular mortality was attributed to the progressive disease known as atherosclerosis (1). Despite extensive research efforts over the past eight decades, the exact cause(s) of atherosclerosis remain largely unknown (2). Recently, however, atherosclerosis has been viewed as a "multifaceted disease produced by several etiologic agents acting by various pathogenetic mechanisms" (2). Modern trends in atherosclerosis research have focused on the study of macromolecular changes in the arterial wall which occur very early in life and which may gradually lead to atherosclerotic involvement (1, 2).

At the cellular level, transformation of normal aorta smooth muscle cells into lipid-laden foam cells is an important event in atherogenesis (3, 4). It has been suggested that aorta cells of susceptible individuals may have a metabolic defect which predisposes such individuals to atherosclerotic involvement (5). This defect has been associated with the inability of arterial cells to metabolize lipid overloads (6) and to regulate lipid transport (5).

Central to consideration of factors initiating atherosclerotic involvement is the metabolism of cholesterol and its esters (7, 8) since these lipids accumulate in arterial intima

in the very early stages of atherosclerosis (9) and increase dramatically as the lesions become more severe (10, 11). Over 70 percent of the cholesterol present in foam cells is in the esterified form (3), and the processes leading to the accumulation of cholesteryl ester in arterial cells have been studied extensively by many investigators.

Although intact arterial tissue has been used in many of these studies (12-14), an increasing number of investigators have used cell cultures to study cholesterol metabolism and its relation to atherogenesis (9, 15, 16). Cell cultures enable investigators to study mechanisms of cholesteryl ester accumulation in a controlled, experimental system isolated from dietary, blood, nervous or hormonal factors which may affect the intact animal. In addition, metabolic irregularities may become more pronounced in cultured cells because of their increased metabolic requirements (17).

Atherosclerosis-susceptible White Carneau and atherosclerosis-resistant Show Racer pigeons provide a useful experimental animal system to study the accumulation of free and esterified cholesterol in aorta tissue (17, 18). In muscular foci at the celiac bifurcation of the aorta and in primary aorta cell cultures, White Carneau smooth muscle cells accumulate significantly more cholesteryl ester than do Show Racer cells (17). In addition, White Carneau cells in culture undergo a sequence of degeneration to lipid-laden foam cells which resembles that occurring in White Carneau aortas during atherogenesis, while similar Show Racer cells differentiate into mature smooth muscle

cells (19).

The objectives of this investigation were: 1) to trace the developmental sequence of cholesteryl ester accumulation in the aortas of young White Carneau and Show Racer pigeons; 2) to determine the cell and extracellular matrix cholesteryl ester fatty acid composition of White Carneau and Show Racer aorta cultures; and 3) to study the metabolism of cholesterol - 4 - ^{14}C by aorta cell cultures from both breeds of pigeon.

In these studies it was important to separate free and esterified cholesterol from other lipid classes prior to measurements of the mass, fatty acid composition, or radioactivity in each. Therefore, a chromatographic procedure was developed which permitted the rapid separation of cholesterol and cholesteryl ester from other lipids on one thin-layer chromatogram (Part I).

With this technique, along with fluorometric quantitation (20) and gas-liquid chromatographic analysis (21), the focal accumulation of cholesteryl esters at the celiac bifurcation of pigeon aortas was described as a function of age (Part II). For both breeds the increase in total cholesteryl ester in celiac foci with age was associated with a distinct pattern of fatty acids esterified to cholesterol.

Since the separation of cellular lipid from extracellular lipid in intact aorta tissue is equivocal, aorta cell cultures were used to examine the types of cholesteryl esters which accumulate in cells and in the extracellular matrix (Part III). Both White Carneau and Show Racer cells had cholesteryl ester fatty

acid patterns which were distinctly different from those of the extracellular matrix and the culture medium. These patterns have been interpreted to suggest the origin of cholesteryl esters in each fraction.

In radioisotope studies of cholesterol metabolism in White Carneau and Show Racer aorta cell cultures, the mass of cholesteryl ester in White Carneau cells was found to increase throughout the culture period examined, but rates of incorporation of labeled cholesterol and accumulation of labeled cholesteryl ester were similar for cells from both breeds of pigeons. However, the extracellular matrix of Show Racer cells accumulated more newly-synthesized cholesteryl - 4 - ^{14}C ester than did the matrix of White Carneau cells. These results are discussed and correlated with various reports in the literature to suggest that accumulation of cholesteryl esters in White Carneau cells may be the result of slower excretion and/or slower relative catabolism.

The manuscript presented as Part I (Separation of Six Lipid Classes on One Thin-layer Chromatogram) has been published in the Journal of Chromatography (22). Part II (Cholesteryl Esters of Pigeon (Columba Livia) Aortas as a Function of Age) will be submitted to Comparative Biochemistry and Physiology while Part III (Cholesteryl Ester Fatty Acid Composition of Aorta Cells Cultured from White Carneau Pigeons) and Part IV (Cholesterol - 4 - ^{14}C Metabolism in White Carneau and Show Racer Pigeon Aorta Cell Cultures) will be submitted, respectively, to The Journal of Clinical Investigation and Experimental and Molecular Pathology.

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PART I

SEPARATION OF SIX LIPID CLASSES ON ONE THIN-LAYER CHROMATOGRAM

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INTRODUCTION

Since the concentrations of squalene[1] and cholesteryl esters[2] in the aorta have been related to the severity of atherosclerosis, it seems highly desirable to be able to separate these two lipid classes, along with triglycerides, fatty acids, sterols, and complex lipids, on one thin-layer chromatogram. Such a separation would allow the six lipid classes to be analyzed fluorometrically or densitometrically in situ on one chromatogram[3] and prevent losses of microgram quantities of these two lipids when scraped, eluted, and rechromatographed for separation by conventional procedures. Previously, Blank et al.[4] separated five lipid classes on a single chromatogram. However, an overlap of the sterol ester and hydrocarbon fractions necessitates the scraping of this region followed by elution and development in a different solvent system (e.g. diethyl ether-petroleum ether (b.p. 38.5-40.0°), 7:93) if these two classes are to be studied. This communication describes a technique for the separation of six lipid classes on one chromatogram through the sequential use of two solvent systems.

MATERIALS AND METHODS

Standard Lipid Mixture

Ten milligrams of the following lipid standards were dissolved in methylene chloride-methanol (2:1) to yield a final concentration of 1 µg of each per µl of solution: L-α-lecithin dipalmitoyl from Schwarz-Mann, Orangeburg, N. Y., cholesteryl oleate and triolein from Applied Science Laboratories, State College, Pa., cholesterol from Steraloids Inc., Pawling, N. Y., palmitic acid from Calbiochem, San Diego, Calif., and squalene from K & K Laboratories Inc., Plainview, N. Y.. If the separated lipids were to be quantitated fluorometrically, the standard solution was hydrogenated by the method of Farquhar et al.[5] to prevent quenching of spots[3].

Thin-layer Chromatography

Merck chromatoplates (Darmstadt, G.F.R.) (20X20 cm) with a 0.25 mm layer of silica gel G were pre-washed in Solvent I (diethyl ether-glacial acetic acid-petroleum ether (b.p. 38.5-40.0°), 100:3:97) and activated at 110° for 30 min. Fifteen microlitres of the standard lipid mixture was spotted at the origin (2 cm above the plate bottom) with a 50 µl Hamilton syringe and repeating dispenser (Hamilton Pb-600). Warm air blowers were used to dry the chromatoplates between sample drops in order to minimize the spot size.

After Solvent I had migrated to 9 cm above the plate bottom, the plate was removed from the chamber and allowed to air dry. The chromatoplate was next placed in Solvent II (diethyl ether-petroleum ether (b.p. 38.5-40.0°), 3:97) in a second chamber. Solvent II was allowed to migrate to 15 cm above the plate bottom. The chromatoplate was then air dried and sprayed with Rhodamine 6 G (ref.6) to visualize the separated lipid classes.

RESULTS AND DISCUSSION

Fig. 1A shows the development of the lipid standards in Solvent I. In this solvent system the phospholipid (1), sterol (2), and free fatty acid (3) separate as individual spots while the triglyceride, sterol ester and hydrocarbon migrate together forming spot 4. Subsequent development in Solvent II results in a further separation of the latter three lipids into individual spots as seen in Fig. 1B. The substantial distance between each lipid class separated by this method permits in situ fluorometric or densitometric analysis of each class or elution and recovery of squalene and cholesteryl esters when radioactive lipids are fractionated.

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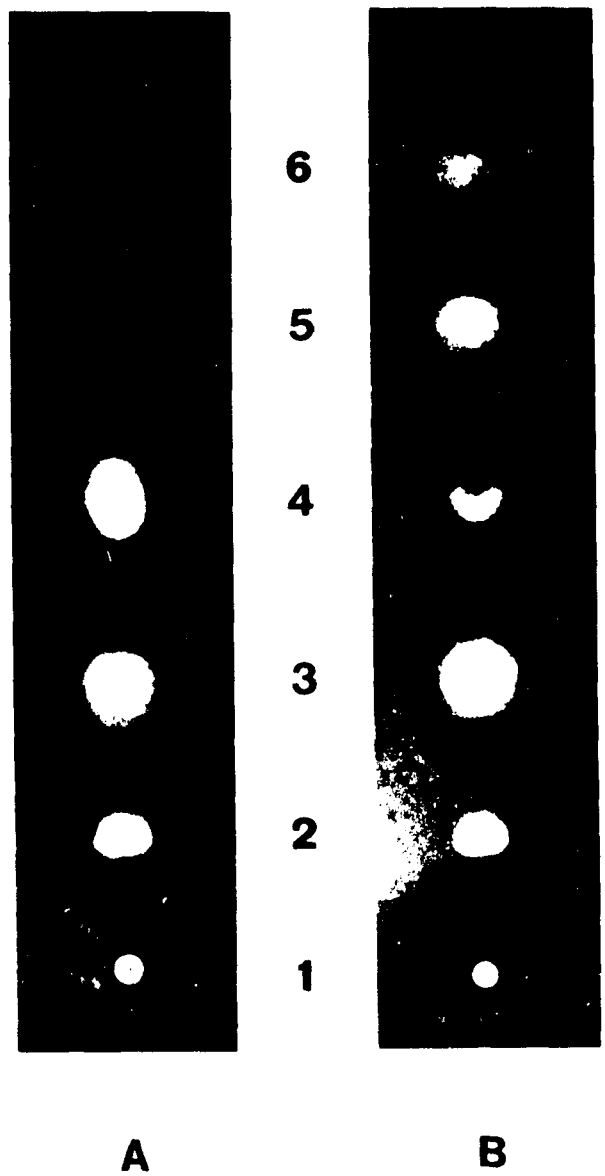

Fig. 1 (A) Thin-layer chromatogram of lipid standards developed in Solvent I (1 = phospholipid at origin; 2 = cholesterol; 3 = free fatty acid; 4 = triglyceride, cholesteryl ester, and squalene). (B) Thin-layer chromatogram of lipid standards after subsequent development in Solvent II. 1 = phospholipid at origin; 2 = cholesterol; 3 = free fatty acid; 4 = triglyceride; 5 = cholesteryl ester; 6 = squalene.

PART II

CHOLESTERYL ESTERS OF PIGEON (*COLUMBA LIVIA*) AORTAS AS A FUNCTION OF AGE

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Abstract--1. The amount and fatty acid composition of cholesteryl esters from upper thoracic aortas and from muscular foci at the celiac bifurcation in 1-day-old, 6-week-old, and 6-month-old White Carneau and Show Racer pigeons (Columba livia) were determined.

2. There were no significant differences in the amounts or composition of cholesteryl esters from upper thoracic aortas between breeds or as a function of age.

3. The nearly linear increase in total cholesteryl ester content in celiac foci was highly correlated with age.

4. Changes in the fatty acids of cholesteryl esters from celiac foci as a function of age were similar for both breeds.

5. Accumulation of saturated cholesteryl esters accounted for the increase in total cholesteryl esters from 1 day to 6 weeks of age.

6. A high correlation existed between the increase in total cholesteryl esters and the increase in cholesteryl oleate and cholesteryl linoleate during the 6-week to 6-month period.

7. Concurrent with the increase of cholesteryl linoleate in celiac foci after 6 weeks of age was an increase in the ratio of polyunsaturated to saturated cholesteryl ester fatty acids.

8. The pattern of cholesteryl ester accumulation in celiac foci of young pigeons resembled that seen in the aortas of other young animals including humans.

INTRODUCTION

White Carneau and Show Racer pigeons have been widely used an an experimental animal system to study aortic lipid accumulation in atherosclerosis. Several investigators (Santerre et al., 1972; Lauper et al., 1975) have made extensive studies of lesion development in these pigeons ranging in age from embryos to 8 years. Santerre et al. (1972) found no breed differences in the incidence, location, developmental history or histopathology of early aortic lesions. However, by 6 years of age lesions were much less severe in Show Racer pigeons than in White Carneaux.

Paired muscular intimal thickenings occur at the celiac bifurcation of the aorta in embryos and squabs of both breeds. By 4-6 months of age lipid became visible in these muscular foci, and their cholesteryl ester content was much greater than that of the upper thoracic aorta (Santerre et al., 1972; Nicolosi et al., 1972). Although cholesteryl ester is the predominant lipid accumulating in atherosclerotic lesions (Portman, 1970), some investigators (Smith and Slater, 1972) have speculated that cholesteryl ester may accumulate in normal aortas as the result of an aging process. Nevertheless, with increasing age, severe lesions become predictably localized at the celiac bifurcation in White Carneaux (Santerre et al., 1972).

The fatty acid composition of cholesteryl esters from aortas of 8-month, 1-year, and 5 to 8-year-old pigeons has been

previously described (Young et al., 1964; Young and Middleton, 1966; St. Clair et al., 1968) but since the earliest visible lipid deposits appear in muscular foci in White Carneaux and Show Racers by 4-6-months of age (Santerre et al., 1972), it is important to examine aorta cholesteryl ester fatty acids of younger pigeons. Such data can define the developmental sequence of cholesteryl ester accumulation at the celiac bifurcation and may indicate the origin of aorta cholesteryl esters.

This communication describes the cholesteryl ester content and the cholesteryl ester fatty acid composition of upper thoracic aortas and celiac foci from 1-day-old, 6-week-old, and 6-month-old White Carneau and Show Racer pigeons. The developmental sequence of aorta cholesteryl ester accumulation is discussed in terms of processes which contribute to the accumulation of specific esters in pigeons, man and other species.

MATERIALS AND METHODS

One-day-old (0-24 hr posthatch), 6-week-old, and 6-month-old White Carneau and Show Racer pigeons were used in this study. Eggs obtained from our own colonies were incubated and provided the source of 1-day-old birds. Six-week-old and 6-month-old pigeons were purchased from Palmetto Pigeon Plant, Sumter, South Carolina. Birds from both sources were derived from inbred lines described by Clarkson et al. (1959).

Birds were sacrificed by exsanguination and samples of the upper thoracic aorta and celiac foci were excised and prepared as described previously (Nicolosi et al., 1972). Four pools of aorta tissue for each site, age, and breed were analyzed for lipid. Each pool contained pieces of aorta tissue from 1-40 birds of both sexes as needed to provide at least 9 mg wet weight.

Following homogenization of aorta tissue in 1 ml of physiological saline (pH 7.0), aliquots were taken for DNA analysis (Prasad et al., 1972) and lipid was extracted by the method of Folch et al. (1957). The final lipid extract was dissolved in 10 ml of methylene chloride : methanol (2 : 1 v/v). A 2 ml aliquot was separated into lipid classes by thin-layer chromatography (Hojnacki and Smith, 1974) and total cholesteryl ester content was determined by in situ fluorometry (Nicolosi et al., 1971). Cholesteryl esters in the remaining 8 ml of lipid extract were separated in a similar manner and eluted from the silica gel (Goldbrick and Hirsch, 1963). Methyl esters were prepared by

direct micro-methanolysis using boron trichloride gas (Lavoie, 1971). Qualitative and quantitative analyses were performed according to established gas-liquid chromatographic procedures (McMullin et al., 1968; Lavoie, 1971). The column was calibrated with Applied Science (State College, Pa.) fatty acid standard K-102 and agreed with the composition data with a relative error less than 10% for major components (> 10% of the total mixture) and less than 10% for minor components (< 10% of the total mixture). Standard fatty acid methyl esters showed a linear response over the range of sample sizes analyzed.

Only 4 higher fatty acids (more than 12 carbons) were quantitated since minor fatty acids eluted between laurate (C_{12}) and arachidonate ($C_{20:4}$) constituted less than 1% of the total fatty acids esterified to cholesterol. Arachidonate was occasionally detected in cholesteryl esters from large pools of aorta tissue. Since in these instances this acid also constituted less than 1% of the total cholesteryl ester fatty acids, it was not included in tabulations of the data.

An analysis of variance was used to determine effects and interactions for breed, age, and aorta site means. For each aorta site Duncan's multiple-range test was used to detect significantly different age means.

RESULTS

No significant age or breed differences in the amount or fatty acid composition of cholesteryl esters from upper thoracic aortas were apparent. Age-related changes in the cholesteryl ester composition of the celiac foci were similar for both breeds; therefore, data is discussed in terms of developmental trends common to both breeds. Since there were no significant age-breed interactions, data points in Figures 1-3 represent mean values for combined data from White Carneau and Show Racer celiac foci.

While the amount of total cholesteryl ester in the upper thoracic aortas of 1-day-old, 6-week-old, and 6-month-old pigeons remains constant, there is an increase in total cholesteryl ester in celiac foci with age ($r = 0.983$) (Fig. 1). Mean values for cholesteryl ester in 1-day-old, 6-week-old, and 6-month-old celiac foci are significantly different ($P < 0.05$).

Although no developmental changes occur in the cholesteryl ester fatty acid composition of the upper thoracic aorta, distinct trends in the accumulation of cholesteryl esters are evident in the celiac foci with age (Fig. 2). There is a rapid increase in the amounts of saturated cholesteryl esters (palmitate and stearate) during the first 6 weeks of life. A high correlation exists between the increase in total cholesteryl ester (Fig. 1) during this 6-week period and the increase in cholesteryl palmitate ($r = 0.989$) and cholesteryl stearate ($r = 0.990$). Subsequently, no significant increase occurs in the amount of saturated esters

during the 6-week to 6-month period.

In contrast, there is no significant increase in the monounsaturated (cholesteryl oleate) and polyunsaturated (cholesteryl linoleate) cholesteryl esters during the first 6 weeks (Fig. 2), but, thereafter, they increase rapidly so that by 6 months the amount of these esters is significantly greater ($P < 0.05$) than the 1-day and 6-week concentrations. Interestingly, there is a high correlation between the increase in total cholesteryl ester (Fig. 1) and the increase in cholesteryl oleate ($r = 0.978$) and cholesteryl linoleate ($r = 0.959$) after 6 weeks of age.

The polyunsaturated to saturated cholesteryl ester fatty acid ratio (PUFA/SAT) in celiac foci remains constant during the first 6 weeks but then rises sharply so that at 6 months it is significantly greater ($P < 0.05$) than the 1-day and 6-week ratios (Fig. 3). An extremely high correlation ($r = 1.000$) exists between the increase in the PUFA/SAT ratio and the increase in cholesteryl linoleate (Fig. 2) during the 6-week to 6-month period.

DISCUSSION

The data presented in Figure 1 confirms earlier reports (Young et al., 1964; Nicolosi et al., 1972; Subbiah et al., 1974) that cholesteryl esters accumulate focally in the celiac bifurcation of pigeon aortas. Since the cholesteryl ester content of celiac foci is greater than the upper thoracic aortas for both breeds at all ages studied (Fig. 1), this accumulation of cholesteryl esters at branch points in the arterial system may reflect a difference in the vascular architecture, as well as associated hemodynamic irregularities (Santerre et al., 1972) at these sites.

The increase in cholesteryl ester content (Fig. 1) in muscular foci for both breeds is highly correlated with age ($r = 0.983$). Some investigators have suggested that accumulation of cholesteryl esters in normal intima may be a benign process related to aging and not associated with atherogenesis (Smith and Slater, 1972). Smith (1965) studied cholesteryl ester accumulation in normal aorta intima from humans ranging in age from 10 to 65 years, and reported that, while the increase in cholesteryl ester in lesion-free intima is highly correlated with age, there is no correlation between this increase and the severity of atherosclerosis. In studies of children aged several hours to 10 years, Day and Wahlqvist (1970) also noticed a high correlation between the increase of cholesteryl esters in normal intima and age.

In the studies by Smith (1965) and Day and Wahlqvist (1970), the upper thoracic aorta was analyzed for cholesteryl ester. The absence of change as a function of time in the cholesteryl ester composition of the upper thoracic aortas of pigeons in the present study (Fig. 1) may reflect the relatively short time intervals considered, as well as the young age of the birds. In contrast, changes with age in the amount and fatty acid composition of cholesteryl esters in the celiac foci (Fig. 1) may reflect accelerated lipid metabolism in this portion of the aorta (Nicolosi et al., 1972; Subbiah et al., 1974).

Data presented in Figure 2 indicates that there is a distinct sequence in accumulation of specific cholesteryl esters in the celiac focus with age. The predominance of saturated cholesteryl esters in the aortas of young pigeons (1 day to 6 weeks) is probably the result of in situ synthesis of palmitic and stearic acid with subsequent esterification to cholesterol (St. Clair et al., 1968). Thereafter, aorta cholesteryl esters begin to assume a pattern of unsaturation resembling that of blood cholesteryl esters (Young and Middleton, 1966; Young, 1969). After 6 weeks this probably results from increased aorta synthesis of cholesteryl oleate along with increased infiltration and deposition of plasma lipoproteins rich in cholesteryl linoleate (St. Clair et al., 1968; Velican, 1974). The PUFA/SAT ratio (Fig. 3) also reflects the change from accumulation of saturated cholesteryl esters to the accumulation of polyunsaturated esters.

This sequence of aorta cholesteryl ester accumulation in

young pigeons appears characteristic of other young animals including humans (Day and Wahlqvist, 1970; Portman, 1970). Böttcher and Woodford (1962) reported that early in life aorta cholesteryl esters were highly saturated but gradually assumed the unsaturation pattern characteristic of serum. Substantial percentages of cholesteryl palmitate and cholesteryl stearate have been found in normal aortas from humans aged 0 to 1 month (Day and Wahlqvist, 1970), 6 months to 20 years (Smith, 1974), and from fetal Rhesus monkeys (Portman, 1970). With increasing age, in all of these studies, there was a decrease in the percentage of saturated aorta cholesteryl esters, an increase in the percentage of cholesteryl linoleate, and a concomitant increase in the PUFA/SAT ratio.

Since pigeon serum contains predominantly polyunsaturated cholesteryl esters (Young and Middleton, 1966; Subbiah et al., 1974), it is unlikely that the saturated and monounsaturated esters accumulating in celiac foci (Fig. 2) originated from the blood unless a highly selective uptake was occurring. Pigeon aortas do, however, have the metabolic capability for de novo fatty acid synthesis, chain elongation, desaturation, and cholesterol esterification (St. Clair et al., 1968). Substantial amounts of radioactivity appear in the palmitic, stearic and oleic acids esterified to cholesterol when White Carneau and Show Racer aortas are perfused with acetate - 1 - ^{14}C (St. Clair et al., 1968). Numerous radioisotope studies with pigeons (St. Clair et al., 1968), monkeys (St. Clair et al., 1969), rabbits (Dayton

and Hashimoto, 1970), and humans (Chobanian and Manzur, 1972) also confirm that in situ synthesis is an active process contributing to the cholesteryl oleate present in arterial tissue. In addition, significant amounts of saturated cholesteryl esters are synthesized by pieces of rat aorta incubated with cholesterol - 4 - ^{14}C (Felt and Beneš, 1969), by rabbit aortas following the intravenous injection of acetate - 1 - ^{14}C (Newman et al., 1968), and by intima-media preparations of rabbit aorta incubated with (U - ^{14}C) palmitic acid (Morrison et al., 1974).

Many investigators (Smith et al., 1967; Velican, 1974) have suggested that the appearance of cholesteryl linoleate in aortas indicates a deposition of plasma lipoproteins since the arterial wall is unable to synthesize linoleic acid (Velican, 1974). It is likely that this deposition in pigeon aortas is augmented after 6 weeks of age. Apparently the PUFA/SAT ratio continues to increase with age since in aortas from 12-15-month-old White Carneau and Show Racer pigeons the ratios are 0.84 and 1.32 respectively (St. Clair et al., 1968), and in 5-8-year-old White Carneau aortas the ratio is 1.98 (Young et al., 1964).

A transition in synthesis from saturated to monounsaturated cholesteryl esters and increased accumulation of cholesteryl linoleate in celiac foci after 6 weeks cannot be explained at the present time. However, since none of the changes occur in the upper thoracic aorta, sequential changes in celiac foci cholesteryl ester composition may be the result of metabolic features unique to branched portions of aortas as suggested by Nicolosi et al. (1972). Furthermore, it would appear that fatty acid

metabolism is an active process in focal areas of pigeon aortas (Subbiah et al., 1974).

In summary, the pattern of cholesteryl ester accumulation in celiac foci of young pigeon aortas resembles that seen in the aortas of other young animals including humans. It is suggested that by 6 weeks of age a change occurs in the pattern of synthesis of cholesteryl esters resulting in diminished amounts of saturated esters and increased amounts of monounsaturated esters appearing in the muscular foci. Concurrently, an increase occurs in the accumulation of cholesteryl linoleate which probably results from increased infiltration and deposition of plasma lipoproteins.

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Fig. 1. Cholesteryl ester content of upper thoracic aortas (○) and celiac foci (●) from 1-day-old, 6-week-old, and 6-month-old pigeons (Columba livia). One-day data points represent the mean of 4 analyses expressed in µg cholesteryl ester/µg DNA. Six-week and 6-month data points each represent the mean of 8 analyses.

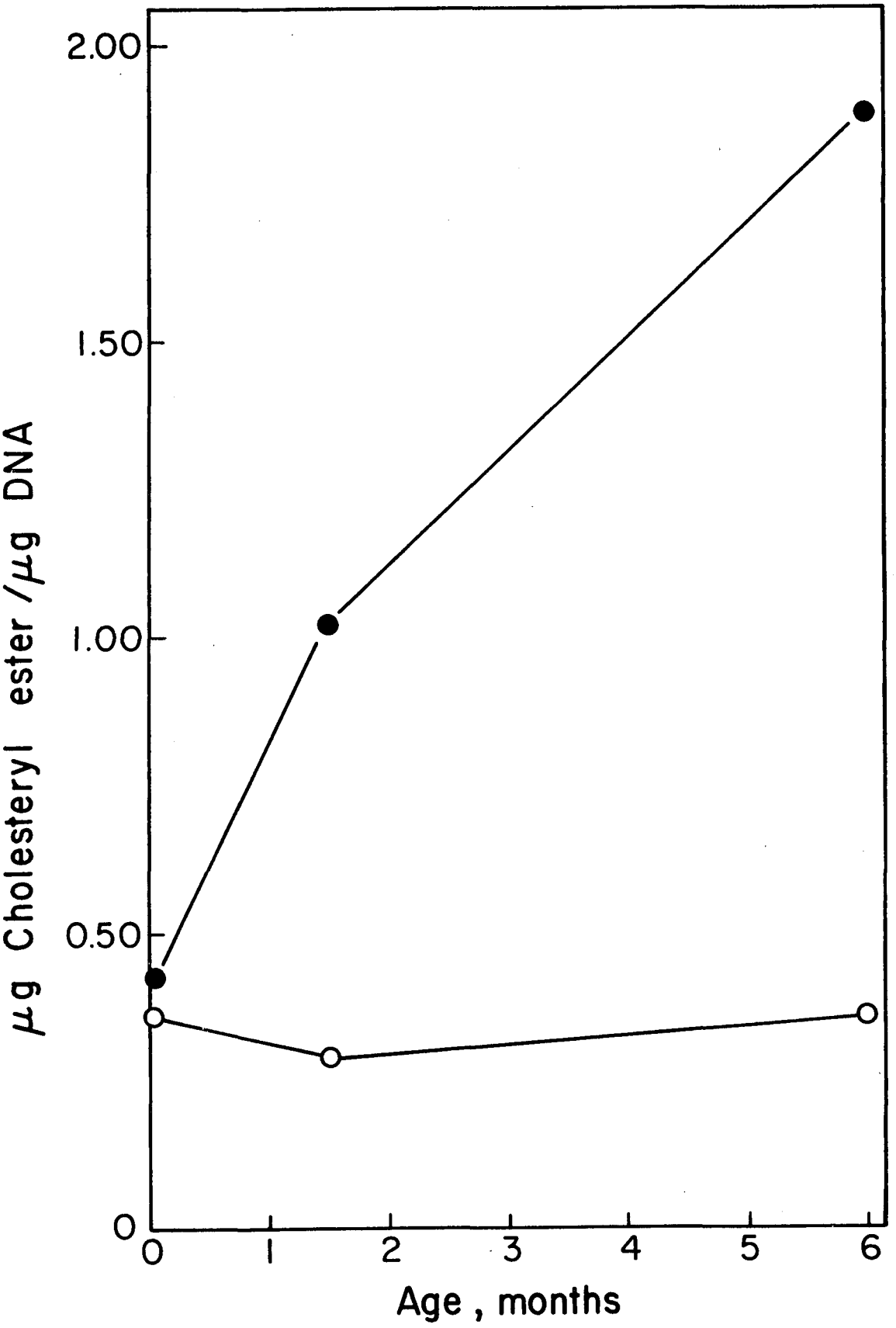

2.00
1.50
1.00
0.50
0
μg Cholesteryl ester /μg DNA
0
1
2
3
4
5
6
Age , months

Fig. 2. Cholesteryl ester fatty acid composition of celiac foci from 1-day-old, 6-week-old, and 6-month-old pigeons (Columba livia). One-day data points represent the mean of 4 analyses expressed in µg fatty acid/µg DNA. Six-week and 6-month data points each represent the mean of 8 analyses. Palmitate (C_{16}) □ ; stearate (C_{18}) Δ ; oleate ($C_{18:1}$) ■ ; linoleate ($C_{18:2}$) ▲ .

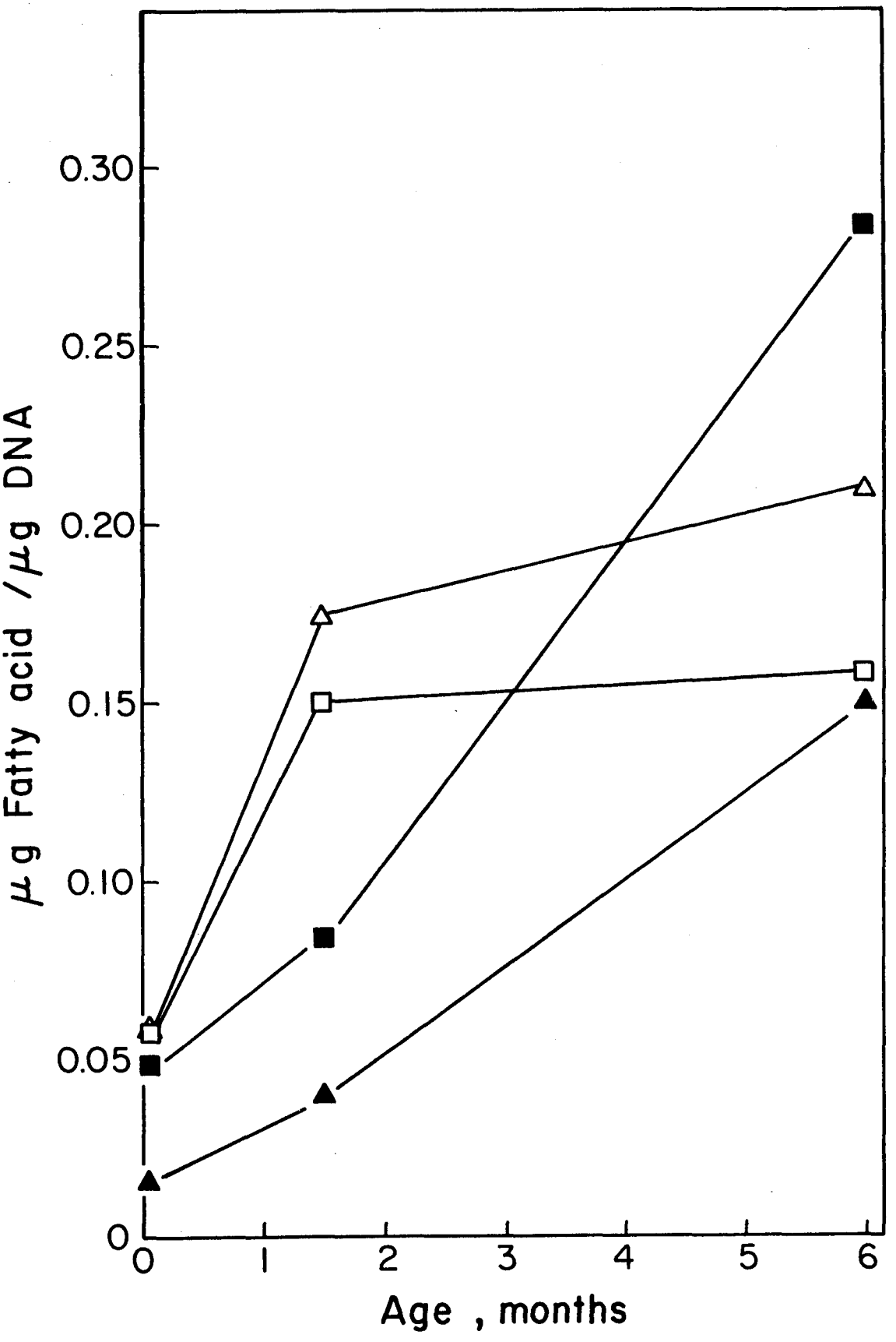
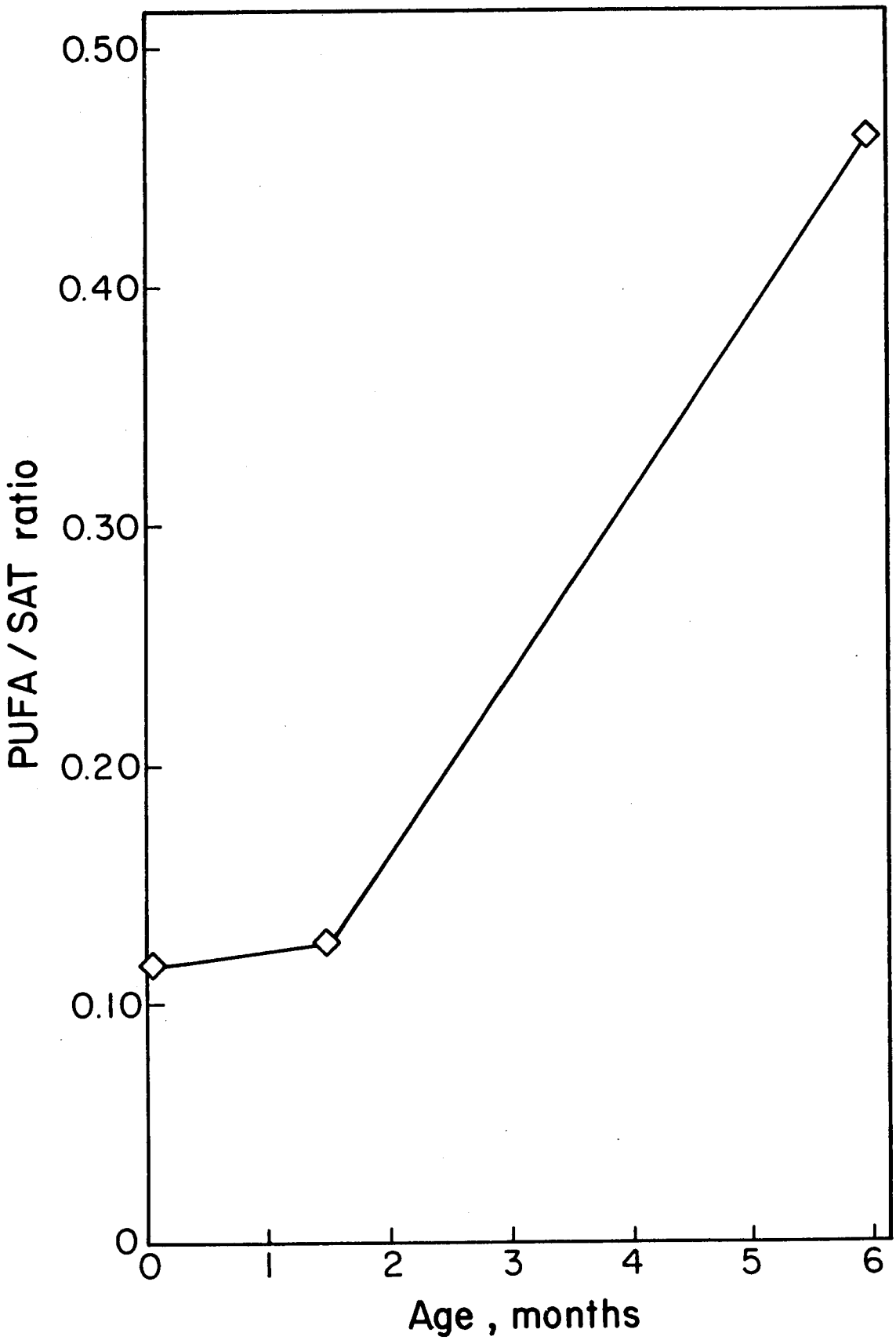

0.30
0.25
0.20
0.15
0.10
0.05
0
μg Fatty acid / μg DNA
0
1
2
3
4
5
6
Age , months

Fig. 3. Ratio of polyunsaturated to saturated cholesteryl ester fatty acids (PUFA/SAT) in aorta celiac foci from 1-day-old, 6-week-old, and 6-month-old pigeons (Columba livia). The 1-day data point represents the mean of 4 analyses while the 6-week and 6-month data points each represent the mean of 8 analyses.


0.50
0.40
0.30
0.20
0.10
0
PUFA / SAT ratio
0
1
2
3
4
5
6
Age , months

PART III

CHOLESTERYL ESTER FATTY ACID COMPOSITION OF AORTA CELLS CULTURED FROM WHITE CARNEAU PIGEONS

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ABSTRACT

Aorta cell cultures from atherosclerosis-susceptible White Carneau pigeons were fractionated into cell, extracellular matrix and culture medium fractions. The amounts of cholesterol and cholesteryl ester and the cholesteryl ester fatty acid composition in each fraction were determined. Both the cell and matrix fractions had higher esterified/free cholesterol ratios than the culture medium. However, the pattern of cholesteryl ester fatty acids in the matrix fraction resembled that of the culture medium while the cell fraction was distinctly different from both the matrix and medium fractions. It is suggested that White Carneau cells in culture may form cholesteryl esters by in situ esterification while the extracellular matrix may bind and concentrate esters from the culture medium.

INTRODUCTION

The accumulation of cholesteryl esters within arterial intima appears to be a key event in atherogenesis (1,2). Based on fatty acid composition, the origin of cholesteryl esters within foam cells of human fatty streaks has been attributed to in situ esterification of cholesterol while infiltration of the arterial wall by plasma lipoproteins is believed responsible for extracellular deposition of cholesteryl esters in fibrous lesions (3). Numerous studies have also suggested that the deposition of lipoproteins in arterial intima may be the result of complex formation with extracellular matrix components (4-6). Glycosaminoglycans appear to be able to bind β- lipoproteins (7), and in vitro preparations of plaque elastin selectively sequester cholesteryl esters from certain lipoprotein fractions (8).

In several postmortem studies (3,9,10) of human aortas, cholesteryl ester fatty acid patterns have been used as an index of the type of lesion and to indicate the predominant location of lipid. Subcellular fractions rich in cholesteryl ester which is present in anisotropic and isotropic forms have also been isolated from fatty streaks and fibrous plaques (11,12). However, in none of these studies has there been an unequivocal separation of cellular from extracellular lipid.

White Carneau pigeons provide an excellent model (13) for studies of cholesteryl ester accumulation since they develop

atherosclerotic lesions which contain significant amounts of cholesteryl ester (14) and which closely resemble lesions seen in humans (15). Several investigators have also shown that the progressive development of atherosclerosis in these pigeons on atherogenic diets is correlated with the amount of cholesteryl esters accumulating in their aortas (16). Aorta cell cultures from White Carneau pigeons accumulate significant amounts of cholesteryl esters (14) and contain predominantly smooth muscle cells which ultrastructurally resemble cells present in sites of lesion formation in White Carneau pigeon aortas (17). In addition, White Carneau smooth muscle cells in vitro undergo a sequence of degeneration to lipid-containing foam cells which resembles that occurring in pigeon and human aortas during atherogenesis (17, 18).

Aorta cell cultures also provide a unique way to study the accumulation of cellular and extracellular cholesteryl esters and, thus, to gain inferential knowledge concerning the origin of these esters. White Carneau pigeon aorta cultures synthesize an extracellular matrix consisting of collagen precursors, immature elastic fibers, chondroitin sulfates A, B, and C, heparan sulfate and hyaluronic acid (17) which have molecular weights ranging from 5000-285,000 (19-21).

This communication describes differences between the cholesteryl ester fatty acid composition of fractions of White Carneau aorta cell cultures. These differences are discussed in terms of processes which may contribute to cholesteryl ester accumulation. Comparisons are also made between the cholesteryl

esters of these culture fractions and the cholesteryl esters present in human serum and aortas.

METHODS

Aorta cell cultures from portions of embryonic thoracic aortas of White Carneau and Show Racer pigeons were prepared as described previously (22) and allowed to propagate for 15 days. Prior to harvesting, the culture medium was decanted, and monolayers were washed with warm, phosphate-buffered physiological saline (pH 7.0) to remove excess medium adhering to cell surfaces. Cell and extracellular fractions were removed from Falcon flasks by gentle scraping with a rubber policeman in 6 ml of buffered saline and transferred to a 1.0 ml calibrated microhomogenizer tube (Anderson Glass Co., Fitzwilliam, N. H.). Empty culture flasks were then stained with Giemsa's stain and examined microscopically (1000X) to confirm that cells and matrix had been completely removed.

After centrifugation for 30 minutes at 2700g, the supernatant in the microhomogenizer tube was transferred with a Pasteur pipette to an XM-300 Centriflo Filter Cone (Amicon Scientific Division, Lexington, Mass.). This filter retains materials with molecular weights equal to or greater than 300,000. By forcing the supernatant through this cone with a centrifugal force of 1000 xg, buoyant and fragile lipid-laden cells were retained in the cone tip (23) while extracellular matrix components containing sequestered lipid passed into the filtrate. This separation of the culture system into three fractions (cell, extracellular matrix and culture medium) is similar to that described by Danes and Bearn (24).

The residue in the cone tip was then removed in buffered saline and added to the cell pellet in the microhomogenizer tube. Following homogenization, aliquots were taken for DNA analysis (25), lipid was extracted by the method of Folch, Lees and Sloane-Stanley (26), and lipid classes were separated by thin-layer chromatography (27). In situ fluorometric quantitation (28) was used to determine the total cholesteryl ester content. Cholesteryl esters were eluted from the silica gel (29), and methyl esters were prepared by direct micro-methanolysis using boron trichloride gas (30).

Qualitative and quantitative analyses were performed according to established gas-liquid chromatographic procedures (30,31). The column was calibrated with Applied Science (State College, Pa.) fatty acid standard K-102 and agreed with the stated composition data with a relative error less than 5% for major components (> 10% of the total mixture) and less than 2% for minor components (< 10% of the total mixture). Standard fatty acid methyl esters showed a linear response over the range of sample sizes analyzed.

For analysis of medium and matrix respectively, 1 ml of culture medium and the centriflo membrane cone filtrate containing the extracellular matrix components were lyophilized. Lipid in the residues was subjected to extraction and analyses as described for cell lipid. An analysis of variance was used to determine whether cell and extracellular matrix means were significantly different. Culture medium means were compared with means of the cell and matrix fractions by Dunnett's test (32).

RESULTS

As indicated in Tables I and II the culture medium has significantly more total cholesteryl ester and more of each cholesteryl ester fatty acid than the cell fraction. The cell fraction in turn has more of each of these lipid components than the matrix fraction. The matrix fraction, however, resembles more closely the culture medium in cholesteryl ester fatty acid composition than the cell fraction (Table II). Both the culture medium and the matrix fraction are significantly different from the cell fraction in the relative percentage of linoleate, in the mean relative percentage of saturated cholesteryl ester fatty acids and in the ratio of polyunsaturated to saturated cholesteryl ester fatty acids. The only similarity between the cell and matrix fraction is their high esterified to free cholesterol ratio which is greater than that of the culture medium (Table I).

In similar aorta cultures from atherosclerosis-resistant Show Racer pigeons, the esterified to free cholesterol ratios of the cell fraction (3.35 ± 0.59) and the matrix fraction (4.59 ± 0.25) are significantly lower ($P<0.05$) than corresponding ratios in White Carneau culture fractions (Table I). Although White Carneau aorta cultures contain significantly more total cholesteryl ester than Show Racer cultures (14), few significant differences exist between breeds in the cholesteryl ester fatty acid composition of cell and extracellular matrix fractions.

Arachidonate ($C_{20:4}$) was detected only in cholesteryl

esters from large pools of Show Racer aorta cultures. In these instances this acid constituted approximately 2% of the total cholesteryl ester fatty acids present. In previous analyses of total fatty acids (22), arachidonate was present in Show Racer aorta cell cultures but absent in White Carneau cultures.

DISCUSSION

Differences between the cell and matrix fractions in amounts and percentages of cholesteryl ester fatty acids (Table II) suggest that the esters in these fractions may have different origins. Although the culture medium has more than sixteen times as much total cholesteryl ester as the cell fraction (Table I), the cells nevertheless maintain a cholesteryl ester fatty acid pattern which is distinctly different from that of the culture medium. More than 50% of the cholesteryl ester fatty acids in the cell fraction are saturated while those of the culture medium are predominantly polyunsaturated.

While White Carneau aorta cells in culture do not seem to passively incorporate the major cholesteryl esters to which they are exposed, they do seem to accumulate esterified cholesterol in preference to the free form. This is evidenced by their high esterified to free cholesterol ratio (Table II). In situ esterification may therefore contribute to the presence of saturated cell cholesteryl esters. This idea is substantiated by observations that: 1) cells in culture are relatively impermeable to cholesteryl ester uptake (33); 2) fatty acid biosynthesis and subsequent cholesterol esterification have been shown to contribute to the cholesteryl esters present in White Carneau aortas (16, 34); 3) after perfusion of White Carneau pigeon aortas with acetate-1-^{14}C, the greatest percentage of radioactivity in the

cholesteryl ester fraction appears in palmitic acid (34, 35); and 4) cholesterol esterification is increased in cell-free preparations of White Carneau aortas following cholesterol feeding (36).

In contrast to the cell cholesteryl esters, the cholesteryl ester fatty acid pattern of the matrix fraction resembles that of the culture medium. This is most evident in the relative percentages of linoleate, the mean relative percentages of saturated cholesteryl ester fatty acids and the polyunsaturated to saturated fatty acid ratios (Table II). Furthermore, these fatty acid relative percentages and the polyunsaturated to saturated fatty acid ratios are significantly different from the cell fraction. The cholesteryl esters sequestered by the extracellular matrix may thus result from a binding and concentration of culture medium lipid.

The patterns of cholesteryl esters in these aorta culture fractions in some respects resemble those reported by Smith, Evans and Downham (9) for human serum and aortas with either predominantly extracellular or predominantly intracellular lipid. Nearly a fourfold difference exists between cell and matrix cholesteryl ester content in White Carneau cultures (Table I). Ultrastructural examination of White Carneau aorta cultures confirms that most of the lipid is located intracellularly (17). Similarly, Smith et al. (9) found that there was a much greater concentration of cholesteryl esters in human intima containing fat-filled cells when compared to intima containing extracellular lipid droplets.

Both human serum and aortas with extracellular lipid have higher relative percentages of linoleate and higher polyunsaturated to saturated fatty acid ratios than aortas with intracellular lipid (9). A similar pattern is evident for culture medium, matrix and cell fractions of White Carneau aorta cultures (Table II). Serum from White Carneau pigeons has also been shown to have a higher relative percentage of linoleate and a higher polyunsaturated to saturated fatty acid ratio than White Carneau aorta tissue (37-39). These similarities suggest that White Carneau aorta cultures might be used as an in vitro model of the human cardiovascular system for studying the origin of intracellular and extracellular aorta cholesteryl esters.

The high esterified to free cholesterol ratios of the White Carneau cell and matrix fractions (Table I) appear to have pathological relevance since significantly lower ratios are found in corresponding fractions of aorta cultures from atherosclerosis-resistant Show Racer pigeons. High esterified to nonesterified cholesterol ratios have been associated with the development of human fatty streaks and are related to cholesteryl ester synthesis (40).

The present study suggests that both cell and extracellular matrix fractions of White Carneau aorta cultures concentrate cholesteryl ester, but probably by different mechanisms. While in situ esterification may contribute to the presence and characteristic pattern of cell cholesteryl esters, selective binding and concentration of medium lipid may be major processes operative in extracellular cholesteryl ester deposition.

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TABLE I

Cholesteryl Ester Composition of Fractions of White Carneau Aorta Cultures

	Culture Medium	Cells	Extracellular Matrix
Total Cholesteryl Ester	141.98 ± 20.91[a,b]	8.44 ± 0.96[b]	2.36 ± 0.28[b]
Esterified Cholesterol to Free Cholesterol Ratio	2.53 ± 0.49[c,d]	13.19 ± 5.14[d]	10.74 ± 4.52

[a] Values are means ± SEM for 4 culture pools expressed in μg cholesteryl ester / μg cell DNA.

[b] Values for each fraction significantly different, $P < 0.05$.

[c] Values are mean ratios of esterified cholesterol to free cholesterol ± SEM from 4 culture pools.

[d] Cells ≠ culture medium, $P < 0.05$.

TABLE II

Cholesteryl Ester Fatty Acid Composition of Fractions of White Carneau Aorta Cultures

	Culture Medium	Cells	Extracellular Matrix
Cholesteryl Ester Fatty Acids			
Palmitate (C_{16})	11.43 ± 1.43[a,b]	1.07 ± 0.17[b]	0.20 ± 0.02[b]
	(18.86 ± 2.38)[c]	(29.94 ± 4.73)	(19.70 ± 2.38)
Stearate (C_{18})	4.22 ± 0.40	1.00 ± 0.13	0.20 ± 0.05
	(6.95 ± 0.66)	(27.79 ± 3.51)	(19.72 ± 4.90)
Oleate ($C_{18:1}$)	13.41 ± 1.41	0.74 ± 0.19	0.22 ± 0.05
	(22.09 ± 2.30)	(20.43 ± 5.30)	(21.77 ± 4.87)
Linoleate ($C_{18:2}$)	31.62 ± 1.24	0.79 ± 0.14	0.39 ± 0.08
	(52.11 ± 2.01)[e]	(21.85 ± 3.96)[e]	(38.81 ± 7.48)[e]
Polyunsaturated to Saturated Fatty Acid Ratio	2.14 ± 0.28[d,e]	0.42 ± 0.08[e]	1.35 ± 0.49[e]
Relative Percentage of Saturated Cholesteryl Ester Fatty Acids	12.91 ± 2.30[f,g]	28.87 ± 2.83[g]	19.71 ± 2.60[g]

TABLE II--Footnotes

[a] Values are means ± SEM from 6 culture pools expressed in μg fatty acid/μg cell DNA.

[b] Corresponding micrograms of each fatty acid different between fractions, $P < 0.05$.

[c] Values in parentheses are corresponding relative percentages ± SEM.

[d] Values are mean ratios of polyunsaturated to saturated cholesteryl ester fatty acids ± SEM from 6 culture pools.

[e] Culture medium ≠ cells; matrix ≠ cells, $P < 0.05$.

[f] Values are mean relative percentages of saturated cholesteryl ester fatty acids ± SEM from 6 culture pools.

[g] Cells ≠ culture medium; cells ≠ matrix, $P < 0.05$.

PART IV

CHOLESTEROL - 4 - ^{14}C METABOLISM IN WHITE CARNEAU AND SHOW RACER PIGEON AORTA CELL CULTURES

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ABSTRACT

Aorta cell cultures from atherosclerosis-susceptible White Carneau and atherosclerosis-resistant Show Racer pigeons were incubated for 48 hr in culture medium containing cholesterol - 4 - ^{14}C. Aliquots of these cultures were harvested at 12 hr intervals, fractionated into cell and extracellular matrix fractions, and the mass and radioactivity of free and esterified cholesterol in each fraction determined.

A linear increase in the mass of cholesteryl esters in White Carneau cells throughout the 48 hr culture period was the only significant change in composition. Rates of incorporation of labeled cholesterol and accumulation of labeled cholesteryl esters were similar for both White Carneau and Show Racer cells. However, after 12 hr in culture, the extracellular matrix of Show Racer cells accumulated more newly synthesized cholesteryl - 4 - ^{14}C esters than did the matrix of White Carneau cells. Accumulation of cholesteryl esters in White Carneau cells may, therefore, be the result of slower excretion and/or slower relative catabolism.

INTRODUCTION

The transformation of normal aorta smooth muscle cells into lipid-laden foam cells is an important event in cellular atherogenesis (Robertson, 1967; Velican, 1974). Aorta cells cultured from atherosclerosis-susceptible White Carneau and atherosclerosis-resistant Show Racer pigeons provide a pathologically relevant model for studying normal and abnormal aspects of lipid metabolism involved in this transition (Nicolosi, Santerre, and Smith, 1972). By 16 days in culture Show Racer cells begin to differentiate into mature smooth muscle cells while White Carneau cells degenerate to lipid-containing foam cells (Wight, 1972). This degenerative sequence resembles that occurring in White Carneau and human aortas during atherogenesis (Cooke and Smith, 1968). White Carneau aorta cells in vitro and at the celiac bifurcation of the aorta in vivo accumulate significantly more cholesteryl ester than similar Show Racer aorta cells (Nicolosi, Santerre, and Smith, 1972).

Since plasma cholesterol is the source of most arterial cholesterol (Portman, 1970), cellular cholesteryl esters probably originate from incorporation of cholesterol followed by in situ esterification (St. Clair and Lofland, 1971). However, excessive incorporation of intact cholesteryl esters, decreased cholesteryl ester hydrolysis and/or excretion, or increased de novo synthesis of cholesterol followed by esterification may all contribute to

accumulation of cholesteryl esters in aorta cells (Smith and Smith, 1973). Simultaneous examination of all of these processes would require compartmentalized mathematical models (Werb and Cohn, 1971a), kinetic studies (Jensen, 1967), morphological identification of subcellular pools (Werb and Cohn, 1971b), and the use of dual-labeled intermediates (Adams, 1973). Such studies are quite complex and are likely to produce equivocal results. Therefore, in an attempt to circumvent these difficulties, experiments were performed to determine whether incorporation of cholesterol followed by esterification and accumulation of labeled cholesteryl ester contributed significantly to the presence of excessive amounts of cholesteryl esters present in cultured White Carneau aorta cells. With this knowledge available, subsequent experiments can be designed to assess the contributions of other possible mechanisms (incorporation of intact esters, intracellular hydrolysis, excretion, and de novo synthesis) to the intracellular accumulation of cholesteryl esters in aorta cells from atherosclerosis-susceptible pigeons.

This communication describes similarities and differences in cholesteryl ester content and in the incorporation of cholesterol - 4 - ^{14}C and accumulation of cholesteryl - 4 - ^{14}C esters between White Carneau and Show Racer aorta cells during a 48 hr culture period. Results are discussed in terms of metabolic differences which may lead to lipid accumulation in White Carneau cells.

MATERIALS AND METHODS

Aorta cell cultures prepared from embryonic thoracic aortas of White Carneau and Show Racer pigeons (Smith et al., 1965) were allowed to proliferate for 14 days. Approximately twenty 30 ml Falcon flasks, each containing 10 White Carneau or Show Racer explants, were used in each experiment. Four flasks were harvested at the start of the experiment, and the amounts of free and esterified cholesterol were determined. The remaining flasks were incubated at 37°C with culture medium containing cholesterol - 4 - ^{14}C (New England Nuclear, Boston, Mass.), and harvests were made at 12, 24, 36 and 48 hr.

Following inactivation of lecithin: cholesterol acyl transferase (E. C. 2.3.1.43) and cholesteryl ester synthetases in the horse serum and chick embryo extract supplements (Fodor, 1950), 100 ml of tissue culture medium was prepared in a 250 ml Erlenmeyer flask equipped with a magnetic stirring bar. One ml of absolute ethanol containing 50 microCuries of cholesterol - 4 - ^{14}C was then added dropwise under sterile conditions to the culture medium at 37°C. Concentrations of ethanol up to 2.5% by volume have been shown to have no adverse effects on aorta cells in culture (Rutstein et al., 1958). Binding of labeled cholesterol to serum lipoproteins was carried out for 3 1/2 hr at 37°C by gentle stirring of the culture medium. Radioactive cholesterol added to serum in this manner is adsorbed first by low density lipoproteins but then undergoes a rapid exchange

and soon reaches an equilibrium between all lipoprotein classes (Goodman and Shiratori, 1964; Miller, Graet, and Frei, 1973). Prior to the start of the experiment, 1 ml of culture medium was lyophilized and amounts of free and esterified cholesterol along with the radioactivity in cholesterol were determined as subsequently described for cell and matrix fractions.

In an effort to determine whether aorta smooth muscle cells in primary culture release newly synthesized cholesteryl esters to the extracellular matrix, monolayers were washed with buffered physiological saline (pH 7.0) to remove excess medium and then separated into cell and extracellular matrix fractions (Hojnacki and Smith, 1975). Aliquots from cell homogenates were taken for DNA analysis (Prasad et al., 1972), and lipid was extracted from both cell and extracellular matrix fractions by the method of Folch et al. (1957). Cholesterol and cholesteryl ester fractions were separated by thin-layer chromatography (Hojnacki and Smith, 1974), quantitated by in situ fluorometry (Nicolosi, Smith, and Santerre, 1971), and the spots scraped into glass scintillation vials. Radioactive lipid was removed from the silica gel by solubilization in 1 ml of Soluene (Packard Instrument Co., Inc., Downers Grove, Ill.) at 56°C for 24 hr and then dissolved in 15 ml of Liquifluor-toluene cocktail (New England Nuclear, Boston, Mass.). Radioactivity was counted in a Packard TriCarb Liquid Scintillation Spectrometer (Model 3320) for sufficient lengths of time to reduce statistical counting error to less than 1%. Quench corrections were made by automatic external standardization.

Experiments were repeated 4 times for each breed. Mass measurements were expressed as µg lipid/µg cell DNA and radioactivity measurements as DPM/µg cell DNA. Mass data from White Carneau and Show Racer cultures were compared statistically using a *t* test.

Least squares regression plots were used to fit lines to that data which appeared linear. For all fitted regressions presented, F values were significant ($P < 0.05$) and correlation coefficients were greater than 0.900. Significant t values ($P < 0.05$) were used to indicate that slopes of lines were different from zero. For data which did not meet criteria for linearity, individual time points were connected by lines.

RESULTS

The only significant change in composition which occurred during the 48 hr culture period was an increase in the amount of cholesteryl ester in White Carneau cells (Table I). Fig. 1 shows the gradual increase in cholesteryl ester content of White Carneau cells as a function of time.

After the initial rapid increase to 12 hr (Fig. 2), cholesterol - 4 - ^{14}C is incorporated by both White Carneau and Show Racer cells more slowly during the remaining 36 hr in culture. The linear increase in esterified cholesterol (Fig. 3) is similar for both breeds.

White Carneau and Show Racer extracellular matrix accumulates newly-synthesized cholesteryl - 4 - ^{14}C esters at essentially the same rate for 12 and 24 hr respectively (Fig. 4). Thereafter, the constant amount of labeled cholesteryl ester appearing in Show Racer matrix is 2 1/2 times greater than that present in White Carneau matrix.

DISCUSSION

Similar incorporation of labeled cholesterol (Fig. 2), as well as similar accumulation of labeled cholesteryl ester (Fig. 3), in White Carneau and Show Racer cells suggest that these patterns are normal features of cellular lipid metabolism and are not causally related to atherogenesis in White Carneaux. This conclusion is supported by reports that: 1) there is no appreciable difference in incorporation of labeled cholesterol by normal and atherosclerotic segments of pigeon aortas in organ cultures (St. Clair and Lofland, 1971); 2) White Carneau and Show Racer pigeon aortas perfused with labeled cholesterol have similar rates of influx and efflux (Bell, Lofland, and Stokes, 1970); and 3) similar amounts of cholesteryl esters are synthesized by perfused aortas from White Carneau and Show Racer pigeons which have previously been on normal diets (St. Clair, Lofland, and Clarkson, 1968). Similar incorporation of labeled free cholesterol and accumulation of labeled esterified cholesterol might also suggest that membranes of White Carneau and Show Racer cells have the same number of lipoprotein receptor sites (Goldstein and Brown, 1975) although the rates of esterification of cholesterol cannot be deduced from the present data.

Some of the newly-synthesized cholesteryl ester is released into the matrices (Fig. 4), and some enters into the intracellular cholesteryl ester pools. Although neither the White Carneau nor the Show Racer intracellular cholesteryl ester

pool became saturated with label during the 48 hr culture period (Fig. 3), there are breed differences in the mode of entry of cholesteryl ester into these pools. Newly synthesized Show Racer cholesteryl ester enters into the intracellular cholesteryl ester pool by exchange since there is no change in cell cholesteryl ester content (Table I). In contrast, in White Carneau cells labeled cholesteryl ester enters into an expanding intracellular pool since the cholesteryl ester content of White Carneau cells increases during the period of culture examined (Fig. 1). Slower cholesteryl ester catabolism and/or excretion by White Carneau cells relative to Show Racer cells appears to be the most likely explanation for the increase in the intracellular cholesteryl ester pool in White Carneau cells.

Although de novo cholesteryl ester synthesis and incorporation of intact cholesteryl esters cannot be eliminated as factors contributing to cholesteryl ester accumulation in White Carneau cells, it is unlikely that these processes are of major importance. Preliminary experiments with acetate - 1 - ^{14}C indicate that White Carneau and Show Racer cells in culture synthesize similar amounts of both free and esterified cholesterol. Other investigators using both aorta perfusion techniques (Lofland et al., 1965) and minced aorta preparations (Lofland, Clarkson, and Artom, 1960) have also been unable to demonstrate breed differences in the amount of lipid synthesized from acetate - 1 - ^{14}C. In addition, the ratio of polyunsaturated to saturated cholesteryl ester fatty acids in the culture medium is

significantly higher than that of White Carneau and Show Racer aorta cells in culture suggesting that few cholesteryl esters are incorporated intact (Hojnacki and Smith, 1975).

Although examination of excretion of cholesteryl ester was not part of the present experimental design, rates of excretion could be influenced by the mode of intracellular lipid dispersion (Dixon, 1961), extent of intracellular hydrolysis (Rothblat and Kritchevsky, 1967), or the availability of energy supplies (Rothblat et al., 1967).

Storage of cholesteryl ester in White Carneau cells in a globular, visible form as opposed to a dispersed, micellar form, might impair its mobilization (Dixon, 1961). Lipid vacuoles and foam cells have been observed in cultures of White Carneau intimal cells but are rarely found in similar Show Racer cultures (Smith et al., 1966). (Between 65-86% of the cholesterol in vacuoles of cultured human arterial cells is in the esterified form (Rothblat and Kritchevsky, 1968).) Nicolosi et al. (1972) suggested that Show Racer aortas may be able to disperse and excrete lipid more efficiently than White Carneau aortas because of their greater phospholipid content.

Since a micellar substrate is needed for optimum activity of cholesteryl ester hydrolase in acetone powder preparations of pigeon aortas (Kritchevsky and Kothari, 1973), storage of cholesteryl esters in a globular form in White Carneau cells might also result in their decreased hydrolysis. Kritchevsky (1972) reported that White Carneau aortas have a lower ratio of cholesteryl

ester hydrolysis to synthesis than do Show Racer aortas. Other investigators (Peters, Takano, and De Duve, 1973; Goldfischer, Schiller, and Wolinsky, 1975) have associated the accumulation of cholesteryl esters in lysosomes of aorta smooth muscle cells with deficient hydrolase activity and have compared lipid accumulation in the arterial wall with genetic storage diseases (Sloan and Fredrickson, 1972).

Metabolic activity appears to be necessary for excretion of cholesteryl ester (Newman and Zilversmit, 1966); thus, it is possible that enzymatic or energy deficiencies might result in decreased ability of White Carneau cells to excrete cholesteryl esters. Energy producing systems in White Carneau intimal cell cultures (Smith et al., 1966) and in White Carneau aorta tissue (Santerre, Nicolosi, and Smith, 1974) appear to be less efficient than comparable Show Racer systems. In addition, Adams (1973) has speculated that sluggish metabolism might be responsible for the diminished efflux of cholesteryl esters from White Carneau fatty streaks and plaques (Lofland and Clarkson, 1970).

The patterns of appearance of cholesteryl - 4 - ^{14}C ester in the extracellular matrix (Fig. 4) suggest that there may be breed differences in the binding of cholesteryl ester to matrix components. Labeled cholesteryl ester in extracellular matrix must accumulate by exchange processes since there is no net change in the cholesteryl ester content of either White Carneau or Show Racer matrix over the 48 hr culture period (Table I). Exchange occurs in two distinct phases, an initial rapid exchange

followed by a much slower phase (Fig. 4).

Although two non-mixing compartments appear to be present in both White Carneau and Show Racer matrix pools (Fig. 4), there are differences in the sizes of these compartments. The larger, rapidly-exchanging compartment of the Show Racer matrix attains equilibrium with labeled ester 12 hr later than the smaller, rapidly-exchanging compartment in White Carneau matrix (Fig. 4). The larger, rapidly-exchanging compartment of the Show Racer matrix might, therefore, permit greater removal of cholesteryl ester from Show Racer cells. A larger slowly-exchanging compartment in White Carneau matrix would retain greater amounts of relatively immobile cholesteryl ester in close proximity to the cell surface which might retard efflux of cholesteryl ester from White Carneau cells. This could explain the gradual accumulation of cholesteryl ester in White Carneau cells during the 48 hr culture period (Fig. 1).

The molecular counterparts to the kinetic compartments of the matrix pools may be glycosaminoglycans. Several investigators (Amenta and Waters, 1960; Berenson et al., 1971; Iverius, 1972) have shown that these macromolecules can bind lipid, and the extracellular matrix synthesized by White Carneau aorta cells in culture contains more glycosaminoglycans than similar Show Racer matrix (Wight, 1972). This could account for the greater amount of cholesteryl ester present in the White Carneau matrix as compared with the Show Racer matrix (Table I). Different amounts of various glycosaminoglycans with different affinities

for lipid could explain the differences in compartments between White Carneau and Show Racer matrices.

The present study suggests that while patterns of incorporation of labeled free cholesterol and accumulation of labeled esterified cholesterol are probably not causally related to intracellular accumulation of cholesteryl esters in White Carneau cells, diminished catabolism and/or excretion may contribute to this accumulation. Further explanation for accumulation of cholesteryl esters in White Carneau cells must await radiotracer studies designed to examine rates of cholesteryl ester catabolism and excretion.

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TABLE I

Free and Esterified Cholesterol in Fractions from White Carneau and Show Racer Aorta Cultures

	Cholesterol[a]	Cholesteryl Ester[a]
Cells		
White Carneau	1.57 ± 0.28	7.52 to 10.73[b]
Show Racer	2.45 ± 0.33	8.35 ± 0.94
Extracellular Matrix		
White Carneau	0.29 ± 0.02	2.70 ± 0.19[c]
Show Racer	0.37 ± 0.06	1.83 ± 0.15[c]
Culture Medium	51.04 ± 7.03	115.25 ± 15.81
Culture Medium Specific Activity	13,035 ± 2348[d]	———

[a]Cell and matrix values are means ± SEM of data from 5 time points expressed in µg lipid/µg cell DNA. Culture medium values are means ± SEM of 6 analyses expressed as µg lipid/ml.

[b]Range over 48 hr culture period expressed as µg cholesteryl ester/µg cell DNA. See Fig. 1.

[c]Significantly different, $P < 0.05$.

[d]Value is mean ± SEM of 6 analyses expressed in DPM/µg cholesterol.

Fig. 1. Change in White Carneau cell cholesteryl ester content during 48 hr culture period. Each time point represents the mean ± SEM of 4 analyses expressed in µg cholesteryl ester/µg cell DNA. The line represents the least squares regression plot.

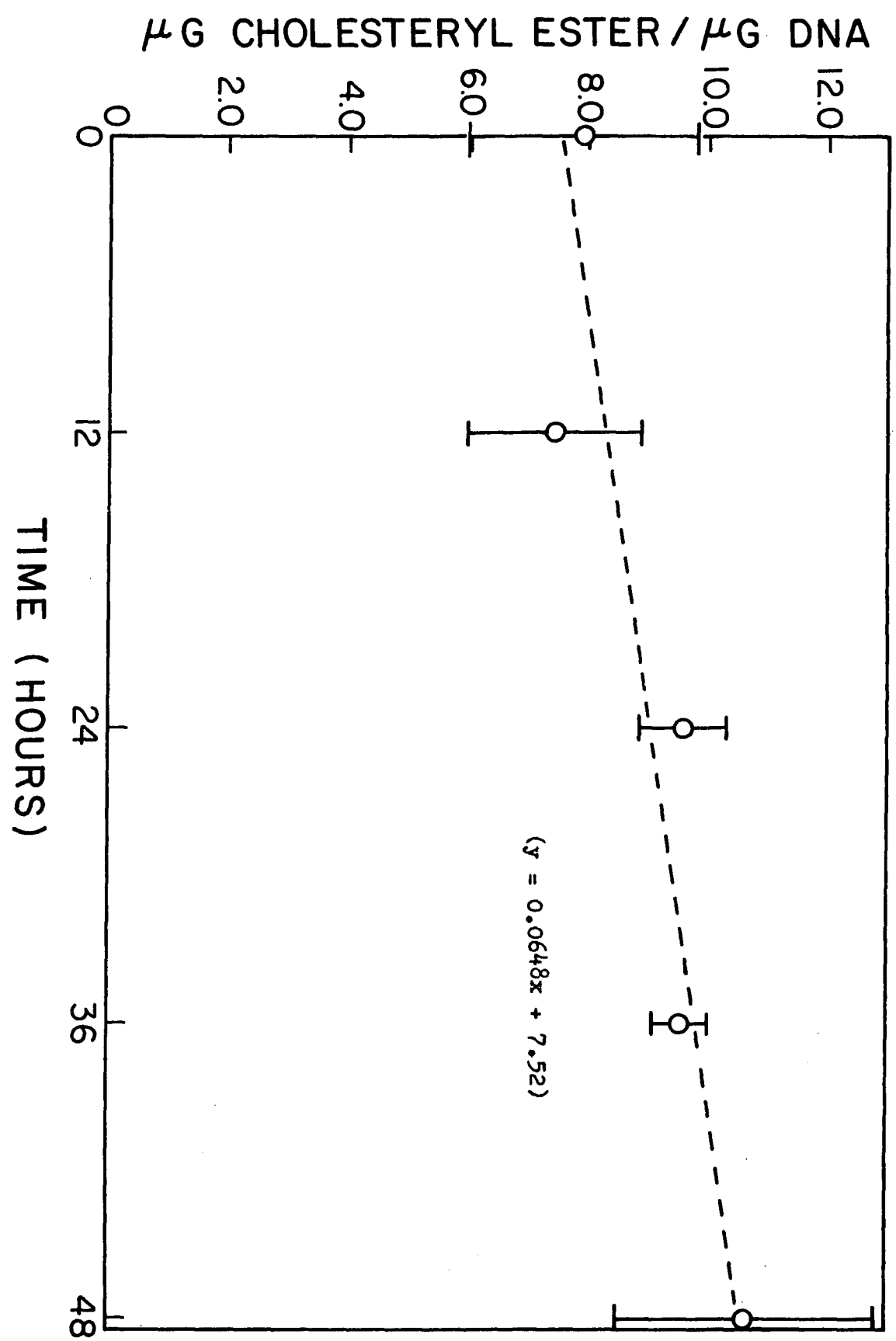

μG CHOLESTERYL ESTER / μG DNA
0
2.0
4.0
6.0
8.0
10.0
12.0
0
12
24
36
48
TIME (HOURS)
(y = 0.0648x + 7.52)

Fig. 2. Incorporation of cholesterol - 4 - ^{14}C by White Carneau (O····O) and Show Racer (●—●) aorta cells during 48 hr culture period. White Carneau and Show Racer aorta smooth muscle cells in primary culture were incubated at 37°C with culture medium containing cholesterol - 4 - ^{14}C, and the amount of labeled free cholesterol present in cells was determined at 12, 24, 36 and 48 hr. Each time point represents the mean of 4 analyses expressed in DPM X 10^3 of cholesterol/µg cell DNA. The line for Show Racer data points from 12 to 48 hr is a least squares regression plot.

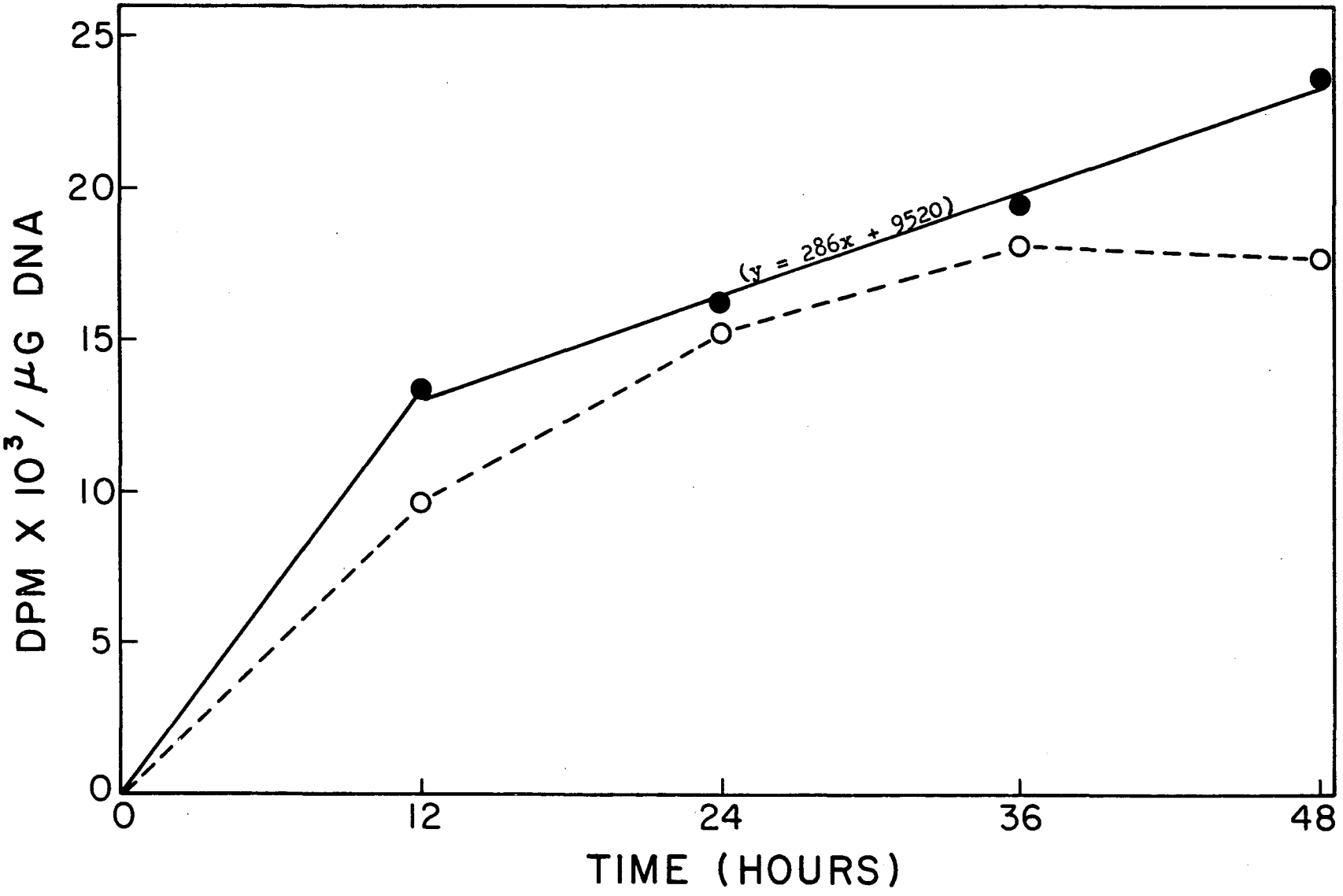

25
20
15
10
5
0
DPM X 10^3 / μG DNA
(y = 286x + 9520)
0
12
24
36
48
TIME (HOURS)

Fig. 3. Accumulation of cholesteryl - 4 - ^{14}C esters in White Carneau (O···O) and Show Racer (●—●) aorta cells during 48 hr culture period. White Carneau and Show Racer aorta smooth muscle cells in primary culture were incubated at 37°C with culture medium containing cholesterol - 4 - ^{14}C, and the amount of cholesteryl - 4 - ^{14}C ester in cells was determined at 12, 24, 36 and 48 hr. Each time point represents the mean of 4 analyses expressed in DPM of cholesteryl ester/μg cell DNA. Lines represent least squares regression plots.

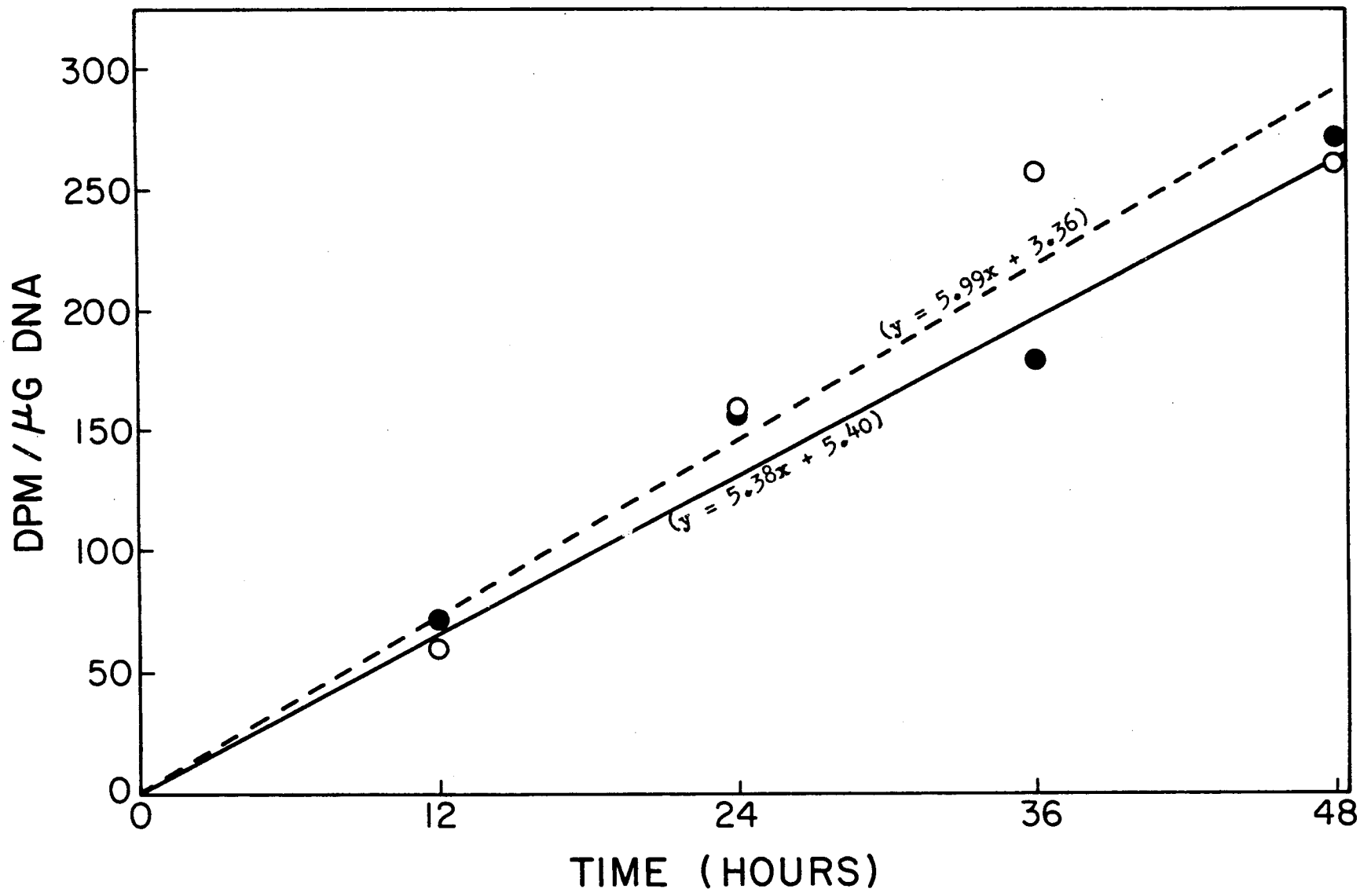
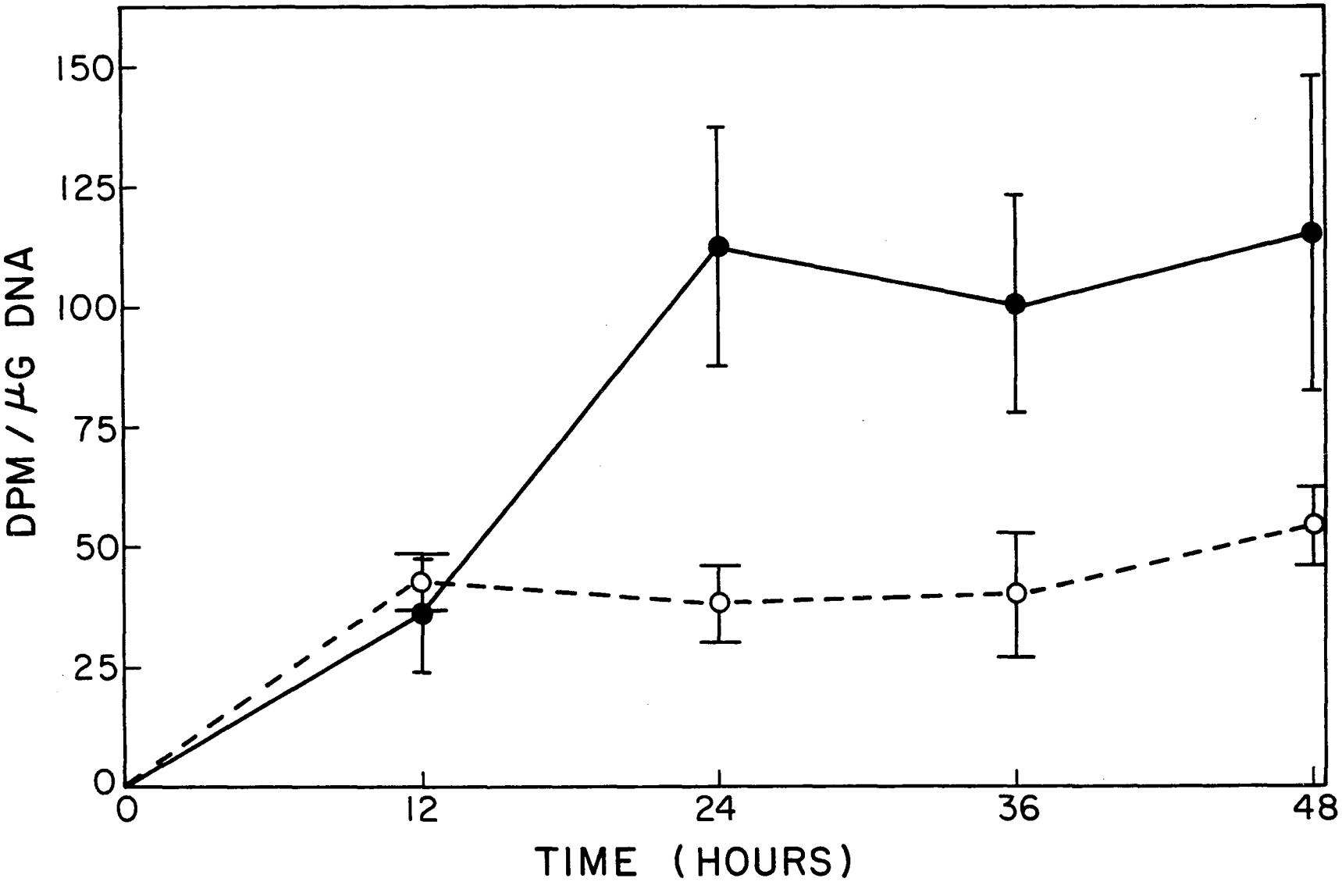

300
250
200
150
100
50
0
0
12
24
36
48
DPM / μG DNA
TIME (HOURS)
(y = 5.99x + 3.36)
(y = 5.38x + 5.40)

Fig. 4. Appearance of cholesteryl - 4 - ^{14}C ester in extracellular matrix of White Carneau (O----O) and Show Racer (●——●) aorta cells during 48 hr culture period. White Carneau and Show Racer aorta smooth muscle cells in primary culture were incubated at 37°C with culture medium containing cholesterol - 4 - ^{14}C. Cultures were separated into cell and extracellular matrix fractions, and the amount of cholesteryl - 4 - ^{14}C ester bound to matrix components was determined at 12, 24, 36 and 48 hr. Each time point represents the mean ± SEM of 4 analyses expressed in DPM of cholesteryl ester/µg cell DNA.


150
125
100
75
50
25
0
DPM / μG DNA
0
12
24
36
48
TIME (HOURS)

BIOGRAPHICAL DATA

Name in Full	Jerome Louis Hojnacki
Date of Birth	March 9, 1947
Place of Birth	Stamford, Connecticut
Secondary Education	Stamford High School Stamford, Connecticut

Collegiate Institutions attended	Dates	Degrees
Southern Connecticut State College	1965-1969	B.S.
University of Bridgeport	1969-1971	M.S.
University of New Hampshire	1971-1975	Ph.D.

Honors or Awards

Stamford Education Association Scholarship
Stamford Medical Association Award
Connecticut State Tuition Scholarship
Phi Sigma
Sigma Xi

Publication

Hojnacki, J. L., and S. C. Smith. 1974. Separation of six lipid classes on one thin-layer chromatogram. J. Chromatogr. 90:365-367.

Positions held	Dates
Instructor--Physiology Stamford High School Stamford, Connecticut	1969-1971
Research Assistant University of New Hampshire Durham, New Hampshire	1972-1975