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METABOLISM OF ARACHIDONIC ACID IN CULTURED PIGEON AORTA CELLS AND RELATED STUDIES

MARY LAVOIE KOES

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Keywords
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METABOLISM OF ARACHIDONIC ACID IN CULTURED PIGEON
AORTA CELLS AND RELATED STUDIES

by

MARY LAVOIE KOES
B.S., Rivier College, 1964
M.S., University of New Hampshire, 1966

A THESIS

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Department of Zoology
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June 1971
Date 7/24/71
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ABSTRACT

METABOLISM OF ARACHIDONIC ACID IN CULTURED PIGEON AORATA CELLS AND RELATED STUDIES

by

MARY LAVOIE KOES

Aortic intimal cells cultured from atherosclerosis-susceptible White Carneau pigeons were reported to be deficient in arachidonate when compared with similar cells from resistant Show Racer pigeons. Studies with 18:2-U-14C and 20:4-1-14C revealed that 92% of the labelled arachidonate is derived by direct incorporation and 8% from synthesis via linoleate during a 24-hour period. There were no apparent differences between breeds in incorporation or synthesis of 20:4ω6. Consequently, the basis for a previously reported arachidonate deficiency in White Carneau cells remains obscure. Aorta cells from Show Racer pigeons did demonstrate significantly greater radioactivity in 20:3ω3 which was derived from the recycling of acetate from 18:2-U-14C oxidation. Oxidation of 18:2-U-14C was extensive in cells from both breeds and resulted in the appearance of label in shorter chain
fatty acids, as well as in unsaturated acids of the w3 and w9 series. Aorta cells cultured from female embryos of both breeds incorporated 13.2% more label from 20:4-l-\(^{14}\)C into phospholipid than cells cultured from male embryos which preferentially incorporated 12.5% more label into the triglyceride fraction.

Re-evaluation of the total fatty acid composition of cultured aorta cells from both breeds indicated that there were no significant (P<.05) breed or sex differences in the relative amounts of any fatty acid from C14 to C22:6.

A modified BCl\(_3\) methanolysis procedure was used to determine the fatty acid composition of pigeon aorta cells. The need for modification was based upon a comparison of fatty acid analyses of mouse fibroblasts determined by GLC following either BCl\(_3\) extraction-trans-esterification or Folch extraction and methanol-sulfuric acid esterification. Differences in analyses resulted from differences in esterification, and neither procedure was satisfactory for less than 3.0 mg cells. Gas volume and solvent ratios in the BCl\(_3\) reaction were adjusted to yield quantitative recoveries of fatty acids from micro
samples of a standard lipid mixture. This modified procedure was successfully applied to samples of mouse fibroblasts as small as 0.8 mg. No side reactions were apparent and purification of the methyl esters prior to GLC was not necessary. Direct methanolysis with BCl$_3$ appears advantageous for small biological samples where simplicity of manipulation and completeness of reaction are important.

Certain problems also arose when working with $^{14}$C-radioactively labelled polyunsaturated fatty acids in small tissue samples. Studies of arachidonate-$^1$-$^{14}$C metabolism in cell cultures demonstrated the following: 1) lack of radiochemical purity in commercially available arachidonic acid-$^1$-$^{14}$C; 2) metabolism of linoleic acid-$^1$-$^{14}$C into several products indistinguishable on GLC; and, 3) retention of radioactivity in the GLC system and its elution in subsequent analyses. Recommendations are presented to circumvent or minimize such hazards.
INTRODUCTION

Various theories have been proposed to explain the genesis of atherosclerotic lesions. Some investigators postulate that circulating abnormalities cause accumulation of excess quantities of lipid in cells of the arterial wall (1,2,3), while others suggest that metabolic defects within these cells predispose to atherosclerotic involvement (2,4). It appears likely that a combination of such factors interact to initiate the disease process, but the relative importance of any particular one may be obscured in the presence of the others. Aortic intimal-medial cells cultured from atherosclerosis-susceptible White Carneau and atherosclerosis-resistant Show Racer pigeons provide a system in which circulatory abnormalities may be controlled or eliminated. Morphological changes which occur in vitro, particularly foam cell development, parallel disease pathology in vivo. (Wight, T. N., Cooke, P. H., Santerre, R. F., and Smith, S. C., unpublished).

The primary objective of this investigation was to study the metabolism of arachidonic acid in aorta cell cultures from both breeds of pigeons.
A comparison of the biosynthesis, incorporation and transformation of arachidonate in aorta cell cultures was made to determine the cause of a reported arachidonate deficiency in White Carneau cells (4) (Part I). Inconsistent results from initial biosynthetic studies with 18:2-U-\(^{14}\)C suggested a re-assessment of the boron trichloride transesterification procedure of Peterson et al (5) (Part II). With development of a method for accurate micro-methanolysis, the metabolic studies were continued. However, there were no breed differences in the incorporation or biosynthesis of arachidonic acid; consequently, the basis for the previously reported deficiency in White Carneau cells (4) was not elucidated. Results of this study prompted a re-evaluation of the total fatty acid composition of aorta cells in both sexes of each breed (Part III).

Certain problems which arose when working with \(^{14}\)C radioactive labelled polyunsaturated fatty acids in small tissue samples are illustrated and discussed in Part IV.

The manuscripts presented in Parts I, II and IV will be submitted to Lipids as full-length articles, whereas Part III will be submitted to the same journal as a short communication.
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PART I

ARACHIDONATE METABOLISM IN CULTURED

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IV. Distribution of Radioactivity from 18:2-\(^{14}\text{C}\) in Higher Fatty Acids of White Carneau and Show Racer Aorta Cells. 23
INTRODUCTION

Essential fatty acid deficiency at the level of arterial cells has been suggested to influence development of atherosclerotic lesions (1,2). Aortic intimal cells cultured from atherosclerosis-susceptible White Carneau pigeons (3) were reported to be deficient in arachidonate when compared with similar cells from resistant Show Racer pigeons. This deficiency in White Carneau cells was accompanied by mitochondrial degeneration similar to that found in early human atherosclerosis in vivo (4). Total fatty acid analyses of embryonic intimal tissue from both breeds revealed no differences in amount of arachidonate and, therefore, suggested that propagation of aorta cells in vitro may promote the manifestation of a metabolic defect leading to arachidonate deficiency in cells from susceptible White Carneau pigeons (3).

It is the purpose of this communication to present comparisons of the biosynthesis, incorporation, and transformation of arachidonate in aorta cell cultures from White Carneau and Show Racer pigeons of both sexes.
MATERIALS AND METHODS

Cell Culture

Cell cultures from the aortic intima of 17-day White Carneau and Show Racer embryos were prepared in Eagles' basal medium supplemented with 15% chick embryo extract, 15% horse serum and 0.146 mg L-glutamine as described by Smith et al (3). Since marked sex differences have been demonstrated in essential fatty acid metabolism of rats (5, 6, 7), embryos were sexed prior to culture of their aortic tissue. Cells were grown in three ounce bottles and refed at two-day intervals with nutrient adjusted to pH 7.3. After ten days' proliferation, nutrient containing linoleic acid-\(\text{U}^{14}\text{C}\) (18:2-\(\text{U}^{14}\text{C}\)) or arachidonic acid-\(\text{L}^{14}\text{C}\) (20:4-\(\text{L}^{14}\text{C}\)) (Applied Science Laboratories) (0.1 \(\mu\)c/ml) was added to the cultures. Following a 24 hour incubation period explanted tissue fragments were removed and the cell layer harvested by scraping with a rubber policeman. The cells were washed twice with saline and uniformly dispersed in 1.0 ml buffered saline. A 0.2 ml aliquot was removed for DNA analysis by the microfluorometric method of Kissane and Robins (8).
Lipid Extraction

For studies of lipid classes cells were subjected to extraction with two 5.0 ml portions of 2:1 (v/v) chloroform-methanol (9). The extracted and washed lipid was immediately redissolved in methylene chloride. Radioactivity in the non-lipid fraction was determined from the residue remaining from filtrations.

Thin Layer Chromatography

Lipid extracts were spotted on plates pre-coated with silica gel G (Brinkman) and developed with petroleum ether-diethyl ether-glacial acetic acid, 84:20:2 (v/v). Spots were located by viewing adjacent standards under ultraviolet light after spraying with Rhodamine 6 G. Spots representing lipid classes (phospholipid, sterol, non-esterified fatty acid (NEFA), triglyceride, cholesterol ester, and hydrocarbon) were scraped into glass scintillation vials for determination of radioactivity (10).

Separation of polyunsaturated fatty acids was achieved on plates pre-coated with silica gel impregnated with 5% AgNO₃ (Analtech) and developed in diethyl ether-glacial acetic acid, 5000:1 (v/v). Spots were located by viewing under ultraviolet light after spraying with
2',7'-dichlorofluorescein and identified by comparison with standards (Hormel Institute).

**Micro-methanolysis**

For separation and analysis of total fatty acids cells were transferred to a 10 ml tissue grinder and sedimented from the saline (50 x g). Cells were homogenized for 3 minutes with a teflon pestle in 3.0 ml, 6:1 (v/v) methanol-methylene chloride. Nonadecanoic acid (5.0 ug/ml) (Applied Science Laboratories) and heptadecanoic acid-1-¹⁴C (0.4 uc/ml) (Philips-Duphar), dissolved in methanol, were added to the cell sample as internal standards prior to direct micro-methanolysis with BCl₃ (Koes, M. L., Ph.D. dissertation). Fatty acid methyl esters were isolated by TLC and recovered from the silica gel by scraping and elution with 20.0 ml of 9:1 (v/v) methylene chloride-methanol. Recovery determined with methyl palmitate-1-¹⁴C was 99.8%.

**Gas-Liquid Chromatography**

All gas liquid chromatographic analyses were performed on a Barber-Colman Model 10 chromatograph with a strontium-90 argon ionization detector. The 6 ft. x 0.25 in glass column was packed with ethylene glycol
succinate (14% on 80/90 mesh Anakrom A) and maintained at 187°C with 15 psi inlet pressure (flow rate - 60 ml/min). The range of linear response for the gas chromatographic system was found to be one to fifty micrograms for each fatty acid methyl ester. Gas chromatographic peaks were identified by comparing retention times with reference standards, by carbon numbers (11) determined under similar conditions, by application of the "end carbon chain" system (12) and by collection, hydrogenation (13) and rechromatography. The column was calibrated with NIH Metabolism Study Section methyl ester Mixtures A,B,D and F. Analytical precision established with Mixture D showed a relative error less than 5% for major components (＞5%) and less than 10% for minor components (＜5%). Chromatographic peaks were quantitated by triangulation or by a disc integrator.

Fatty acid methyl esters were recovered for radioactivity determination by passing the effluent gas stream from the argon ionization detector through a heated line (235°C) to a 7.5 cm glass cartridge (5 mm OD) connected to the GLC exit port by a 5 cm teflon tube (1.5 mm OD). The cartridge contained silica gel (2.5 cm) into which the
teflon tube extended (3 mm) and was plugged at both ends
with glass wool. The contents of the cartridge were
eluted with three 5 ml portions of liquifluor (New Eng­
land Nuclear Corp.) into a scintillation vial. The
efficiency of collection determined from 11 samples of
methyl palmitate-1-14C (442 dpm/ug) over a range of 1.0
to 30.0 ug was 80.6±3.9 S.D.

The following fatty acid methyl ester fractions
were collected for determination of radioactivity: 14:0 -
18:1, 18:2, 18:3 - 20:3, 20:4 and 22:6. Radioactivity was
determined in a Packard dual-channel liquid scintillation
spectrometer for lengths of time sufficient to reduce
the counting error to less than 1%. Quench corrections
were made by the channels ratio method (14).

Nutrient Analysis

Duplicate 1.0 ml aliquots of fresh culture
nutrient used in each experiment were lyophilized prior
to lipid extraction. Extracted lipid was subjected to
TLC and the non-esterified fatty acid fraction eluted for
micro-methanolysis and GLC analysis to determine the
specific activity of the radioactive fatty acid in the
culture nutrient.
Statistical Analysis

Differences in the incorporation and transformation of arachidonic acid-1-\(^{14}\)C and in the conversion of linoleic acid-U-\(^{14}\)C by male and female aortic cells from both breeds of pigeons were determined by an F test using analysis of variance of a 2 x 2 factorial balanced for unequal numbers of samples (15).

RESULTS

Incorporation and Distribution of Arachidonate-1-\(^{14}\)C

There are no significant differences (\(P<.05\)) between cells cultured from the two breeds in the amount of label from 20:4-1-\(^{14}\)C incorporated into cellular lipid or non-lipid (Table I) or in the per cent label distributed among the various lipid classes (Table II). The per cent label incorporated from the nutrient was relatively low (Table I) despite the fact that the addition of 20:4-1-\(^{14}\)C increased the amount of 20:4w6 in the nutrient from 0.39 to 0.94 \(\mu\)g/ml. (The increase in total lipid was negligible.) The greatest amount of radioactivity in cells was present in the triglyceride and phospholipid fractions (Table II). The relatively low per cent activity in the NEFA is indicative of rapid
label utilization. Radioactivity in the sterol and hydrocarbon fractions must result from the recycling of acetate-1-^{14}C derived from the degradation of 20:4-1-^{14}C. A portion of the sterol activity may be attributed to trailing from samples migrating ahead of the sterol or to contamination by mono and/or diglycerides. Degradation and recycling of 20:4-1-^{14}C to acetate-1-^{14}C was extensive since approximately 30% of the label incorporated was found in the non-lipid fraction (Table I).

Although there was no apparent breed difference in label distribution, there was a significant sex difference (Table II). Aorta cells from female embryos incorporated 13.2% more label from 20:4-1-^{14}C into phospholipid than cells from male embryos which preferentially incorporated 12.5% more label into the triglyceride fraction.

Conversion of Linoleate-U-^{14}C

In order to derive an estimate of 20:4w6 synthesis, 18:2-U-^{14}C was added to the culture nutrient and the cells incubated for a 24 hour period. Since 20:4w6 cannot be distinguished from 20:3w3 and 22:1w9 on GLC (Koes, M.L., Ph.D. dissertation) the composite fraction was collected and separated by argentation TLC. The relative per cent radioactivity determined for each component is presented in Table III. Show Racer cells demonstrated
significantly greater (P < .05) per cent radioactivity in 20:3w3. Although it is tempting to speculate that breed and sex differences exist for 22:1w9 and 20:4w6 respectively, wide variation, as indicated by the standard errors (Table III) precludes such speculation.

Specific activities of other fatty acid methyl ester fractions collected from GLC are shown in Table IV. The largest amount of radioactivity remained in 18:2w6. Radioactivity in the 14:0 - 18:1 and 22:6 was necessarily derived from recycled acetate-14C.

Origin of Cellular 20:4w6

During a 24 hour incubation period 0.48 nanomoles 18:2-U-14C/ug cell DNA was incorporated (mean of 17 experiments), and 0.018 nanomoles converted to 20:4w6. In the same time period 0.19 nanomoles 20:4-l-14C/ug cell DNA was incorporated (mean of 23 experiments). From these data 10 day old pigeon aorta cells appear to derive 92% of their 20:4w6 from uptake of free fatty acid and 8% from synthesis via 18:2w6. These percentages must be regarded as maximal and minimal respectively since oxidative loss of 20:4w6 biosynthesized from 18:2w6 could not be determined.
DISCUSSION

Similar to aortas of the squirrel monkey (16) and the rabbit (17), pigeon aorta cells in vitro, as well as in the perfused aortas (18), appear to possess the metabolic potential for synthesis of fatty acids via chain elongation and desaturation. There appear to be no metabolic blocks in the conversion of 18:2 to 20:4 similar to that reported by Haggerty et al in Hela cell cultures (19). Pigeon aorta cells, like rat heart cells (20), show active metabolism of linoleic acid leading to the formation of arachidonic acid. It was postulated that the dedifferentiation or transformation of Hela cells (a stable cell line) resulted in loss of the desaturation mechanism (20). Although freshly isolated heart cells can catalyze this conversion their ability to desaturate is also lost after periods of time in culture (20). A comparison of synthetic rates between primary pigeon aorta and primary rat heart cells in vitro is precluded since Harary et al (20) did not attempt to isolate 20:4w6 from other esters of similar retention values. The low per cent synthesis (8%) compared with per cent incorporation (92%) is consistent with the observation of Howard and Kritchevsky (21) that the free fatty acids of tissue culture nutrient are taken up
directly from the serum pool with very little synthesis. Bailey (22) has shown that L cells can synthesize fatty acid but synthesis is inhibited when cells are grown in the presence of a serum.

The significantly greater (P<.05) per cent activity in 20:3w3 of Show Racer cells (Table III) may indicate that a control mechanism is operative. It is well established that higher polyunsaturated fatty acids of the 18:3w3 series inhibit the formation of 20:4w6 and higher metabolites of the 18:1w9 series (23). The lower per cent radioactivity in 22:1w9 of Show Racer cultures may be indicative of such a control, but lack of a difference in 20:4w6, as well as experimental variations, make further interpretation difficult.

Steinberg et al (24) reported that incorporation of 18:2-1-14C into 20:4w6 of several rat tissues was extremely low due to rapid oxidative catabolism of 18:2w6. A considerable amount of degradation also occurs in tissue culture as evidenced by appearance of radioactivity in the 14:0-18:1, 22:1w9, 20:3w3 and 22:6 fractions. Variations in the mean specific activities of these acids probably reflects the extremely variable
nature of lipid oxidation in cultured cells. Cell type as well as the conditions under which the cells are cultured influence oxidation (25). Although smooth muscle cells comprised the majority of the culture populations, small numbers of fibroblasts and macrophages (Wight, T.N., unpublished) may contribute to experimental variation.

The proportion of labelled fatty acid incorporated into lipid classes also seems to be related to conditions under which the cells are cultivated. Cultures, in which more exogenous fatty acids are present than required for phospholipid synthesis or necessary for a carbon and energy source, incorporate excess fatty acids into cellular triglycerides (25). Bailey (26) studied lipid uptake by several cell cultures and concluded that triglyceride was most rapidly taken up, followed by phospholipid and cholesterol. Triglycerides and phospholipids are major constituents of our tissue culture nutrient as well as of the cell culture lipids. Therefore, it appears logical that the largest per cent label from 20:4-1-\textsuperscript{14}C is found in these two lipid classes. The presence and synthesis of hydrocarbons from acetate-\textsuperscript{14}C in the pigeon tissue culture system is consistent with
the study of St. Clair et al (27) in which they demonstrated considerable synthesis of squalene by perfused adult pigeon aortas.

It is surprising that pigeon aorta cells, supplied with embryo extract and serum enriched media, demonstrate a sex difference in the distribution of label from 20:4-1-14C. Estrogens are reported to enhance arterial incorporation of 32P into phospholipid both in vivo and in vitro while testosterone appears to inhibit this process in vitro (28). In our cultures all cells are subjected to a similar chemical environment. However, female sex hormones present in the mare serum of the culture medium may stimulate increased phospholipid synthesis and/or preferential 20:4 incorporation by aorta cells cultured from female embryos. Isolated human, canine and rat arterial tissue have been shown to convert estrone to estradiol as well as testosterone to androstenedione (29). Perhaps pigeon aorta tissue is also capable of such conversions which would potentiate any sex effects in vitro.

It is well established that female rats on essential fatty acid deficient diets are able to maintain higher levels of 20:4w6 in liver phospholipids for a
longer period of time than males (6,7). Sex differences have also been noted in rat-liver microsomal preparations in which the female elongated 16:0 to 18:0 approximately twice as rapidly as the male (5). However, these same investigators were unable to show any sex difference in the conversion of 18:2 to 20:4 or in the incorporation of 20:4 into complex lipids. Their conclusion that no sex differences existed in 20:4ω6 synthesis is not completely warranted since no attempt was made to separate 20:4ω6 from methyl esters of similar GLC retention values.

We were unable to demonstrate any breed differences in the incorporation or synthesis of arachidonate in cultured pigeon aorta cells. Therefore, the role of essential fatty acids in cell function at the mitochondrial level and in atherogenesis in pigeons remains unclear.
ACKNOWLEDGEMENTS

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REFERENCES


TABLE I

Incorporation of Label from 20:4-1\textsuperscript{14}C by Aorta Cells of White Carneau and Show Racer Pigeons

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Grand Mean ± S.E. (dpm/ugDNA)</th>
<th>Sex</th>
<th>White Carneau</th>
<th>Show Racer</th>
<th>Sex mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Lipid</td>
<td>19799 ± 2374</td>
<td>F</td>
<td>18055\textsuperscript{a}</td>
<td>20436\textsuperscript{b}</td>
<td>19246</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>23349\textsuperscript{a}</td>
<td>15116\textsuperscript{b}</td>
<td>20605</td>
</tr>
<tr>
<td>Breed Mean</td>
<td></td>
<td></td>
<td>20879</td>
<td>17776</td>
<td></td>
</tr>
<tr>
<td>Cell Non-Lipid</td>
<td>9067 ± 1188</td>
<td>F</td>
<td>8983</td>
<td>10972</td>
<td>9707</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>8958</td>
<td>7525</td>
<td>8480</td>
</tr>
<tr>
<td>Breed Mean</td>
<td></td>
<td></td>
<td>8970</td>
<td>9249</td>
<td></td>
</tr>
<tr>
<td>Total\textsuperscript{c}</td>
<td>28867 ± 2809</td>
<td>F</td>
<td>27039</td>
<td>31409</td>
<td>28628</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>32308</td>
<td>22641</td>
<td>29085</td>
</tr>
<tr>
<td>Breed Mean</td>
<td></td>
<td></td>
<td>29849</td>
<td>27025</td>
<td></td>
</tr>
<tr>
<td>% Total Label Incorporated</td>
<td>1.4 ± 0.06</td>
<td>F</td>
<td>1.5</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>1.6</td>
<td>1.2</td>
<td>1.4</td>
</tr>
<tr>
<td>Breed Mean</td>
<td></td>
<td></td>
<td>1.6</td>
<td>1.2</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Figures represent means of eight and seven experiments for White Carneau male and female respectively.

\textsuperscript{b} Figures represent means of four experiments each for Show Racer male and female.

\textsuperscript{c} Label lost as CO\textsubscript{2} or as material soluble in Folch partition solvents is not included.
### TABLE II

Distribution of Label From $^{14}$C in Lipid Classes of White Carneau and Show Racer Aorta Cells

<table>
<thead>
<tr>
<th>Lipid Class&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Per cent label in class</th>
<th>Sex</th>
<th>White Carneau</th>
<th>Show Racer</th>
<th>Sex mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipid</td>
<td>35.3 ± 3.6</td>
<td>F&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>42.2</td>
<td>42.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>26.4</td>
<td>29.0</td>
</tr>
<tr>
<td>Breed mean</td>
<td></td>
<td></td>
<td>35.8</td>
<td>34.3</td>
<td></td>
</tr>
<tr>
<td>Sterol</td>
<td>5.4 ± 0.6</td>
<td>F</td>
<td>5.4</td>
<td>4.9</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>6.1</td>
<td>4.7</td>
<td>5.6</td>
</tr>
<tr>
<td>Breed mean</td>
<td></td>
<td></td>
<td>5.8</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>NEFA</td>
<td>7.2 ± 1.3</td>
<td>F</td>
<td>7.9</td>
<td>6.0</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>7.5</td>
<td>6.2</td>
<td>7.1</td>
</tr>
<tr>
<td>Breed mean</td>
<td></td>
<td></td>
<td>7.7</td>
<td>6.1</td>
<td></td>
</tr>
<tr>
<td>Triglyceride</td>
<td>49.1 ± 3.3</td>
<td>F&lt;sup&gt;+&lt;/sup&gt;</td>
<td>42.2</td>
<td>44.3</td>
<td>43.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>53.3</td>
<td>57.6</td>
<td>54.7</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>48.1</td>
<td>50.9</td>
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</tr>
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</table>
TABLE II—Continued

<table>
<thead>
<tr>
<th>Lipid Class&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Grand Mean ± S.E.</th>
<th>Breed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per cent label in class</td>
<td>Sex</td>
</tr>
<tr>
<td>Cholesteryl ester</td>
<td>2.1 ± 0.3</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>Breed mean</td>
<td></td>
</tr>
<tr>
<td>Hydrocarbon</td>
<td>0.9 ± 0.2</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>Breed mean</td>
<td></td>
</tr>
</tbody>
</table>

* Significant sex effect (P < .05)

<sup>a</sup> Lipid extracts were spotted on plates pre-coated with silica gel G (Brinkman) and developed with petroleum ether - diethyl ether - glacial acetic acid, 84:20:2 (v/v).

<sup>b</sup> Figures represent means of eight and seven experiments for White Carneau male and female, respectively.

<sup>c</sup> Figures represent means of four experiments each for male and female Show Racer.
TABLE III

Distribution of Radioactivity From 18:2-U-\(^{14}\)C in Components of GLC Fraction with Retention Value of 3.73\(^a\)

<table>
<thead>
<tr>
<th>Component</th>
<th>White Carneau</th>
<th>Show Racer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male (5)</td>
<td>Female (5)</td>
</tr>
<tr>
<td>20:4w6</td>
<td>27.6(^c)</td>
<td>43.7</td>
</tr>
<tr>
<td>22:1w9</td>
<td>56.4</td>
<td>37.9</td>
</tr>
<tr>
<td>20:3w3(^*)</td>
<td>16.1</td>
<td>18.5</td>
</tr>
</tbody>
</table>

\* Significant breed difference (P<.05)

\(^a\) The composite fraction was collected manually from GLC and separated on silica gel impregnated with 5% AgNO\(_3\) with diethyl ether - glacial acetic acid, 5000:1 (v/v).

\(^b\) Number of experiments

\(^c\) Per cent of total label in fraction collected
TABLE IV

Distribution of Radioactivity from 18:2-U-\(^{14}\)C in Higher Fatty Acids of White Carneau and Show Racer Aorta Cells

<table>
<thead>
<tr>
<th>Fatty Acid Fraction Collected</th>
<th>Male (\times 10^3)(^{b})</th>
<th>Female (\times 10^3)</th>
<th>Mean ± SE</th>
<th>%</th>
<th>Male</th>
<th>Female</th>
<th>Mean ± SE</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-18:1</td>
<td>1317</td>
<td>912</td>
<td>1114 ± 122</td>
<td>10.1(^c)</td>
<td>420</td>
<td>1024</td>
<td>722 ± 178</td>
<td>7.7</td>
</tr>
<tr>
<td>18:2</td>
<td>6586</td>
<td>4257</td>
<td>5421 ± 984</td>
<td>49.1</td>
<td>4502</td>
<td>4255</td>
<td>4378 ± 133</td>
<td>46.8</td>
</tr>
<tr>
<td>&gt;18:2-&lt;20:4</td>
<td>4098</td>
<td>2665</td>
<td>3386 ± 696</td>
<td>30.6</td>
<td>2384</td>
<td>4288</td>
<td>3336 ± 32</td>
<td>35.7</td>
</tr>
<tr>
<td>20:4, 22:1, 20:3</td>
<td>1077</td>
<td>621</td>
<td>849 ± 108</td>
<td>7.7</td>
<td>434</td>
<td>966</td>
<td>700 ± 129</td>
<td>7.5</td>
</tr>
<tr>
<td>22:6</td>
<td>323</td>
<td>234</td>
<td>278 ± 22</td>
<td>2.5</td>
<td>85</td>
<td>354</td>
<td>219 ± 59</td>
<td>2.3</td>
</tr>
<tr>
<td>% Incorporated(^d)</td>
<td>6.6</td>
<td>6.8</td>
<td>-</td>
<td>6.7</td>
<td>8.5</td>
<td>10.2</td>
<td>-</td>
<td>9.3</td>
</tr>
</tbody>
</table>

\(a\) Number of experiments

\(b\) Dpm/ug fatty acid/ugDNA

\(c\) Per cent of total label in fractions collected

\(d\) Per cent of 18:2 in medium incorporated into cells
PART II

DIRECT MICROMETHANOLYSIS OF ANIMAL CELLS

WITH BORON TRICHLORIDE GAS
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INTRODUCTION

Few procedures for methanolysis of microgram quantities of higher fatty acids without prior extraction from biological materials have been reported. Although a number of methods are available for the preparation of methyl esters from lipid extracts (1), many involve the liberation of free fatty acids by hydrolysis or saponification followed by conversion to methyl esters. The methyl esters formed by most methods are subsequently partitioned from methanol to a less polar solvent and washed with water or dilute base to remove the esterification catalyst. Such manipulations decrease the likelihood of quantitative recovery.

Boron trichloride transesterification (2), a one-step extraction-esterification, appears advantageous for small biological samples where simplicity of manipulation and quantitative recovery for GLC are important. In this procedure fatty acid methyl esters may be extracted directly from the evaporated reaction mixture and injected into the column without purification (3).
Although BCl₃ transesterification has several apparent advantages as a micro method, different proportions of fatty acids have been reported from direct transesterification when compared with transesterification of lipid extracted from the same biological material (2). Consequently, fatty acid analyses of cells by BCl₃ extraction - transesterification were compared with analyses of extracted lipid from similar cells following methanol-sulfuric acid esterification.

Since slight differences were found, optimal reaction conditions for BCl₃ micro-esterification of pure lipid mixtures were established and the applicability of these conditions to micro-extraction-methanolysis of lipid in animal cells demonstrated.

EXPERIMENTAL PROCEDURES AND RESULTS

Reagents

All solvents were reagent grade and redistilled prior to use.

Extraction

The method of Folch et al. (4) was employed to
extract and purify lipids from mouse fibroblasts.

Esterification Methods

Methyl esters were prepared from lipid extracts by esterification with methanol-sulfuric acid (100:1, v/v) (5). Samples were refluxed for two hours and diluted with water. The methyl esters were repeatedly extracted with petroleum ether. Combined extracts were washed with water, evaporated under nitrogen and dissolved in methylene chloride for GLC.

The BCl$_3$ transesterification apparatus described by Peterson et al. (2) was modified by the use of teflon tubing from the gas cylinder to the glass gas delivery tube. The volume of BCl$_3$ (Matheson) was measured with an inverted gas buret (containing hexane) modified to provide inlet and outlet connections to the BCl$_3$ cylinder and reaction flask, respectively. The desired amount of BCl$_3$ was measured and delivered to a 25 ml round bottom flask by the pressure of the hexane displaced. Since the measuring apparatus was entirely glass (necessitated by the reactivity of BCl$_3$ with hexane-moist rubber tubing) and, therefore, awkward to manipulate, bubble counts were determined for gas volumes dispensed. Bubble counts were
subsequently used on a routine basis to achieve constant
gas volumes. After the desired amount of BCl₃ was
delivered to the methanolic esterification mixture, reflux-
ing was initiated rapidly and maintained for ten minutes
with a pre-heated mantle (90°C). Moderate agitation was
provided throughout reaction with a glass covered magnetic
stirring bar. The reaction mixture was evaporated at 45°C
in a stream of nitrogen, and the methyl esters extracted
with methylene chloride for GLC analysis.

Rapid esterification reagents, such as BCl₃, have
been reported to produce undesirable side reactions, pro-
ducts from which may mimic methyl esters on GLC (6). How-
ever, reagent blanks run before and after each reaction
series did not give rise to any spurious peaks upon GLC.
Furthermore, in order to prevent cross-contamination
between samples, the glass delivery tube and condenser
were rinsed thoroughly with 2:1(v/v) methylene chloride-
methanol.

Gas-Liquid Chromatography

All gas-liquid chromatographic analyses were
performed on a Barber-Colman Model 10 chromatograph with
a strontium – 90 argon ionization detector. The
6 ft x 0.25 in glass column was packed with ethylene glycol succinate (12% on 80/90 mesh Anakrom A) and maintained at 187°C with 15 psi inlet pressure (flow rate-60 ml/min). The range of linear response for the gas chromatographic system was found to be one to fifty micrograms for each fatty acid methyl ester. Gas chromatographic peaks were identified by comparing retention times with reference standards, by carbon numbers (7) determined under similar conditions and by application of the "end carbon chain" system (8). The column was calibrated with NIH Metabolism Study Section methyl ester Mixtures A, B, D and F. Analytical precision established with Mixture D showed a relative error less than 5% for major components (> 5%) and less than 10% for minor components (<5%). Chromatographic peaks were quantitated by triangulation or by a disc integrator.

Thin Layer Chromatography

Analytical thin layer chromatography was used to determine whether all gas chromatographic peaks obtained by direct methanolysis represented fatty acids.
Samples were spotted on plates pre-coated with silica gel G (Analtech or Brinkman) and developed with petroleum ether-diethyl ether-glacial acetic acid (79:20:1) (9). Spots were located by viewing under ultraviolet light after spraying with Rhodamine B or 6G. Methyl esters were recovered for GLC by the method of Goldbrick and Hirsch (10).

Comparison of Extractions

Low-line mouse fibroblasts (NCTC clone 2555) propagated in medium NCTC 109 for five days were harvested by scraping with a rubber policeman, washed twice by centrifugation (200 x g) from saline and transferred to pre-weighed aluminum dishes for lyophilization and subsequent dry weight determination. Direct methanolation of mouse fibroblasts in 25 ml methanol with BCl₃ was compared with Folch extraction followed by methanol-sulfuric acid esterification over a range of sample sizes (0.070 mg to 25.0 mg). Differences between methods over this range of sample sizes are shown in Table I. Neither method when applied to less than 3.2 mg of cells demonstrated repeatable recovery of methyl esters. A slight chain length effect was also apparent. The Folch/methanol-sulfuric acid procedure
preferentially recovered fatty acids eluted before 16:2; whereas, BCl₃ methanolysis preferentially recovered those eluted after 16:2.

In order to determine whether the differences observed, particularly in C15 and C18, were due to extraction or esterification, extracted lipid from 60 mg of mouse fibroblasts was dissolved in methanol and divided into six equal aliquots. Three samples were esterified in methanol-sulfuric acid and three in BCl₃-methanol. The data in Table II represents two replicate experiments. Differences in esterification parallel the differences of Table I and, therefore, are not a function of lipid extraction. Cl₈ appears to be preferentially esterified by BCl₃-methanol while Cl₁₅ is esterified to a greater extent by methanol-sulfuric acid.

Conditions for Micro-esterification

In order to optimize reaction conditions for micro-transesterification, a pure lipid mixture was prepared in CHCl₃-MeOH (2:1,v/v). It contained 1,2-di-palmitoyl-SN-glycero-3-phosphorycholine (3.0 ug/ml), tristearin (4.3 ug/ml), arachidic acid (3.7 ug/ml) (Mann Research Laboratories) and cholesteryl oleate (3.4 ug/ml)
(Applied Science Laboratories). These four components, each with a different fatty acid, represented the major fatty acid containing lipid classes and permitted estimation of differences in esterification between classes of lipid. The designated purity (99+%) of each lipid was verified by GLC analysis of large (milligram) quantities.

Solvent volumes were reduced to 5.0 ml and a less polar solvent, methylene chloride, was introduced to obtain solubility of the diverse lipid classes. Aliquots (57.6 ug in 4.0 ml) of the standard lipid mixture were taken to dryness under nitrogen in a 25 ml round bottom flask and redissolved in methanol or methanol-methylene chloride, as shown in Table III, to a total sample volume of 5.0 ml. Methanol-sulfuric acid esterification followed by GLC analysis was used to determine appropriate solvent ratios. A 6:1(v/v) ratio of methanol-methylene chloride produced acceptable results (-1.4% maximal mean difference between theoretical and calculated).

With the solvent ratio established, 57.6 ug aliquots of the standard lipid mixture were subjected to BCl₃ trans-esterification. When two grams of gas (2) were delivered to this reaction mixture the methyl ester from cholesteryl
oleate was not recovered by GLC. Since Peterson et al. (2) reported that under conditions sufficient for transesterification no significant structural changes should occur, it was necessary to determine an appropriate gas volume for esterification. Results of micro-esterifications with various gas volumes are shown in Table IV. With less than 0.32 g of BCl₃ gas the esterification of free fatty acid proceeded preferentially at the expense of the phospholipid fraction. When the gas exceeded 0.39 g, methyl oleate, derived from the cholesteryl ester, decreased dramatically. With 0.32 to 0.36 g BCl₃ transesterification was found satisfactory (+1.4% maximal mean difference between theoretical and calculated).

Direct BCl₃ Transesterification of Animal Cells

Conditions established for esterification of the standard lipid mixture were applied to 0.8 and 2.0 mg cells. Mouse fibroblasts, harvested as previously described, were transferred to a 30 ml tissue grinder with 10 ml methylene chloride-methanol (2:1,v/v) and homogenized for 5 minutes with a teflon pestle. The homogenate was divided into equal aliquots of determined dry weight. An internal standard, nonadecanoic acid (C19) (Applied Science
Laboratories) dissolved in methanol (8.0 ug/ml), was added to each aliquot and the total sample volume was adjusted to 5.0 ml maintaining the previously determined solvent ratio (6:1 CH$_3$OH-CH$_2$Cl$_2$). After BCl$_3$ transesterification the mixture was evaporated to dryness under nitrogen, triturated in 10.0 ml methylene chloride and filtered through glass wool previously washed with methylene chloride. After filtration the glass wool was rinsed with methylene chloride and the rinses added to the sample. The solution was concentrated for GLC. Results are shown in Table V. TLC of the esterified biological sample and subsequent GLC of the recovered fatty acid methyl ester spot gave results within ± 2.0% of the original sample as shown in Table V. No peaks were lost after TLC.

DISCUSSION

In this study the criterion for acceptability of agreement between means was arbitrarily established at 2.0% maximum difference (actual percentage composition). This limit was chosen since gas chromatographic analyses are reported to have a precision of ± 1-2% (11). However, much better precision can be obtained between replicate
analyses of standard methyl ester mixtures (12). Because of the various manipulations involved in extraction - methanolysis and the limits of GLC, a maximum difference of ±2.0% for the overall procedure was considered reasonable.

The accuracy and precision of the micro-analytical procedure finally developed were well within these limits (Tables IV and V) and correlated in most cases with the precision expected for replicate analyses of methyl ester standards (12). Since this is the limit of GLC no better precision can be expected from the total analytical procedure.

The present study (Table II) shows that differences (±2.0%) in analyses of extracted lipid between methanol-sulfuric acid esterification and methanolysis with BCl₃ as originally described (2) are the preferential esterifications of C₁₅ and C₁₈ respectively. All other fatty acids are recovered within the 2.0% limit. A trend toward preferential esterification by methanol-sulfuric acid of lower fatty acids (<₁₆) at the expense of higher (>₁₆) and more unsaturated acids is suggested by data in Table I. This trend may be due in part to the longer reaction time of that method (2 hr as opposed to 20 min for BCl₃). Morrison and Smith (13) compared the effects of three commonly used
methanolation reagents - HCl, H\textsubscript{2}SO\textsubscript{4} and BF\textsubscript{3} - on mixtures of unsaturated methyl esters by treatment for 90 min at 100°. Comparable losses of each unsaturated methyl ester were found with all reagents particularly when the duration of treatment or concentration of catalyst increased. Losses were greatest for methyl esters of polyunsaturated fatty acids. This effect may be reflected by the consistently lower per cent recovery of 20:4 following methanol-sulfuric acid esterification in analyses reported here.

It should be noted that excess treatment with BCl\textsubscript{3} also will result in losses of unsaturated acids. More than 0.39 g BCl\textsubscript{3} causes the virtual disappearance of methyl oleate derived from cholesteryl oleate (Table IV). Peterson et al. (2) explained this phenomenon by formation of high boiling polymers through linkages at the double bonds with no elution of such polymers from the column. Lough (14) has shown that BF\textsubscript{3}-methanol also can cause serious losses of 18:1 by formation of methoxy methyl stearate isomers in high yield. This is contrary to general experience (13) and seems to be due to an abnormally high BF\textsubscript{3} concentration (50%, w/v, as opposed to the more common 12.5 to 14%, w/v). With the proper adjustment of catalysts both BF\textsubscript{3} (13) and BCl\textsubscript{3}-methanol yield
satisfactory results.

In addition to adjusting concentration of the esterification catalyst it is also necessary to optimize solvent ratios for complete esterification. As shown in Table III methanol alone is insufficient. It is apparent that cholesteryl oleate is only slightly soluble in methanol as evidenced by the poor recovery of methyl oleate upon GLC. The widely varying solubility characteristics of lipid classes make it imperative to determine appropriate polar - non-polar ratios for complete esterification. With gram quantities Dugan et al. (15) report that the suspension of biological material in ether prior to direct low temperature methylation with methanol-sulfuric acid is essential.

Under the conditions accepted as satisfactory, esterification of triglyceride was greater (Table IV) than that of other lipid classes. With milligram quantities of lipid Morrison and Smith (11) reported methanolysis of tripalmitin to be slower in BCl₃-methanol than in BF₃-methanol due to the formation of appreciable quantities of diglyceride isomers. This effect was not obvious when microgram quantities of lipid were subjected to BCl₃ transesterification in the present study.
Thin layer chromatography of transesterified biological samples demonstrated the completeness of esterification as evidenced by the appearance of large quantities of methylated fatty acids and sterols. All other lipid classes were absent or present in quantities less than 0.25 ug, the lower limit of detection in the TLC system used. It appears that purification of small samples of methyl esters from mouse fibroblasts is unnecessary prior to GLC. However, purification of methyl esters by TLC before GLC may be desirable in certain biological systems. Previous work on a variety of biological tissues also indicated that fatty acid methyl esters may be extracted directly from the evaporated reaction mixture and injected into the column without purification (3).

Boron trichloride transesterification appears to be an acceptable method for the micro-extraction-methanalysis of animal cells. With appropriate operating conditions this procedure provides quantitative recoveries with no loss of unsaturated fatty acids.
ACKNOWLEDGEMENTS

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REFERENCES


### TABLE I

**Differences Between Relative Per Cents Mouse Fibroblast Fatty Acids Recovered by Methanol-H$_2$SO$_4$ Methanolsysis of Extracted Lipid and by Direct BC$_3$-Methanolysis as a Function of Sample Weight.**

<table>
<thead>
<tr>
<th>Dry wt cells (mg)</th>
<th>Fatty Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14:0</td>
</tr>
<tr>
<td>-------------------</td>
<td>------</td>
</tr>
<tr>
<td>20.0 - 25.0</td>
<td>+1.5</td>
</tr>
<tr>
<td></td>
<td>+1.5</td>
</tr>
<tr>
<td>13.0 - 16.0</td>
<td>+1.0</td>
</tr>
<tr>
<td></td>
<td>+0.9</td>
</tr>
<tr>
<td></td>
<td>+0.7</td>
</tr>
<tr>
<td></td>
<td>+0.2</td>
</tr>
<tr>
<td>5.0 - 8.0</td>
<td>-0.2</td>
</tr>
<tr>
<td></td>
<td>+1.0</td>
</tr>
<tr>
<td>3.2 - 3.5</td>
<td>+1.2</td>
</tr>
<tr>
<td></td>
<td>+1.4</td>
</tr>
<tr>
<td>0.07 - 0.75</td>
<td>+1.5</td>
</tr>
<tr>
<td></td>
<td>+3.3</td>
</tr>
<tr>
<td></td>
<td>+1.4</td>
</tr>
<tr>
<td></td>
<td>-1.4</td>
</tr>
<tr>
<td>Mean diff (3.2-25.0 mg)</td>
<td>+0.9</td>
</tr>
</tbody>
</table>

* Folch/methanol- H$_2$SO$_4$ minus BC$_3$-methanol.
# TABLE II

Recoveries of Fatty Acids from Extracted Mouse Fibroblast Lipid Following Methanol-$H_2SO_4$ and $BCl_3$-Methanol Transesterification.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Experiment 1</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14:0</td>
<td>15:0</td>
<td>iso16</td>
<td>16:0</td>
<td>16:1</td>
<td>iso18</td>
<td>16:2</td>
<td>18:0</td>
<td>18:1</td>
<td>18:2</td>
<td>18:3</td>
</tr>
<tr>
<td>Sample #</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Methanol-H$_2$SO$_4$</td>
<td>2.5</td>
<td>3.4</td>
<td>0.0</td>
<td>21.7</td>
<td>2.2</td>
<td>1.4</td>
<td>0.0</td>
<td>23.2</td>
<td>17.7</td>
<td>12.3</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>BCl3-Methanol</td>
<td>1.6</td>
<td>1.1</td>
<td>0.0</td>
<td>19.4</td>
<td>1.3</td>
<td>0.4</td>
<td>0.0</td>
<td>25.4</td>
<td>19.2</td>
<td>13.8</td>
<td>1.4</td>
</tr>
<tr>
<td>Mean</td>
<td>2.5</td>
<td>4.0</td>
<td>0.0</td>
<td>19.7</td>
<td>2.1</td>
<td>1.7</td>
<td>0.0</td>
<td>22.1</td>
<td>18.2</td>
<td>12.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Mean</td>
<td>1.6</td>
<td>1.1</td>
<td>0.0</td>
<td>18.9</td>
<td>1.5</td>
<td>0.4</td>
<td>0.0</td>
<td>27.4</td>
<td>18.2</td>
<td>14.3</td>
<td>0.7</td>
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<tr>
<td>Difference between means</td>
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<td>+3.4</td>
<td>0.0</td>
<td>0.0</td>
<td>+0.6</td>
<td>+1.4</td>
<td>0.0</td>
<td>-3.8</td>
<td>-1.2</td>
<td>-1.4</td>
<td>-0.20</td>
</tr>
</tbody>
</table>
Recoveries of Fatty Acids from Extracted Mouse Fibroblast Lipid Following Methanol-$\text{H}_2\text{SO}_4$ and $\text{BCl}_3$-Methanol Transesterification.

| Experiment | 14:0 | 15:0 | iso16 | 16:0 | 16:1 | iso18 | 16:2 | 16:0 | 16:1 | iso18 | 16:2 | 18:0 | 18:1 | 18:2 | 18:3 | 20:0 | 20:1 | 20:2 | 20:4 |
|------------|------|------|-------|------|------|-------|------|------|------|-------|------|------|------|------|------|------|------|------|------|------|
| 1          | 2.9  | 1.0  | 0.2   | 27.0 | 1.6  | 0.2   | 0.2  | 28.6 | 26.0 | 6.1   | 0.6  | 0.2  | 0.3  | 0.0  | 5.1  |
| 2          | 2.4  | 1.6  | 0.1   | 26.7 | 1.3  | 0.1   | 0.2  | 27.2 | 27.0 | 5.9   | 0.7  | 0.2  | 0.5  | 0.1  | 6.0  |
| 3          | 1.8  | 0.8  | 0.1   | 25.7 | 1.5  | 0.1   | 0.1  | 26.5 | 28.1 | 6.1   | 0.6  | 0.2  | 0.4  | 0.2  | 5.8  |
| Mean       | 2.4  | 1.1  | 0.1   | 26.5 | 1.4  | 0.1   | 0.2  | 28.1 | 27.0 | 6.1   | 0.7  | 0.2  | 0.4  | 0.1  | 5.6  |

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<th>15:0</th>
<th>iso16</th>
<th>16:0</th>
<th>16:1</th>
<th>iso18</th>
<th>16:2</th>
<th>16:0</th>
<th>16:1</th>
<th>iso18</th>
<th>16:2</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>18:3</th>
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<td>0.2</td>
<td>28.3</td>
<td>1.4</td>
<td>0.9</td>
<td>0.0</td>
<td>30.6</td>
<td>24.3</td>
<td>5.2</td>
<td>0.5</td>
<td>0.6</td>
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<td>4.9</td>
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<td>0.2</td>
<td>0.1</td>
<td>24.9</td>
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<td>0.5</td>
<td>0.0</td>
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<td>25.9</td>
<td>5.6</td>
<td>0.8</td>
<td>0.4</td>
<td>0.2</td>
<td>0.2</td>
<td>5.4</td>
<td></td>
<td></td>
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<td></td>
</tr>
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<td>0.3</td>
<td>0.3</td>
<td>25.8</td>
<td>1.3</td>
<td>0.6</td>
<td>0.0</td>
<td>33.9</td>
<td>24.8</td>
<td>5.5</td>
<td>0.5</td>
<td>0.3</td>
<td>0.6</td>
<td>0.2</td>
<td>4.8</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Mean</td>
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<td>0.3</td>
<td>0.2</td>
<td>26.3</td>
<td>1.4</td>
<td>0.7</td>
<td>0.0</td>
<td>32.6</td>
<td>25.0</td>
<td>5.4</td>
<td>0.6</td>
<td>0.4</td>
<td>0.3</td>
<td>0.1</td>
<td>5.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Difference between means

<table>
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</tr>
</thead>
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<tr>
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<td>+0.8</td>
</tr>
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<td>-0.1</td>
</tr>
<tr>
<td>0.0</td>
<td>-0.6</td>
</tr>
<tr>
<td>+0.2</td>
<td>-4.5</td>
</tr>
<tr>
<td>+2.0</td>
<td>+0.7</td>
</tr>
<tr>
<td>+0.1</td>
<td>-0.2</td>
</tr>
<tr>
<td>+0.1</td>
<td>+0.1</td>
</tr>
<tr>
<td>+0.1</td>
<td>+0.0</td>
</tr>
<tr>
<td>+0.5</td>
<td>-0.1</td>
</tr>
</tbody>
</table>

\* Relative per cent.
EFFECT OF SOLVENT RATIO ON TRANSESTERIFICATION OF A STANDARD LIPID MIXTURE WITH METHANOL-H$_2$SO$_4$ DETERMINED BY RECOVERIES OF FATTY ACIDS

<table>
<thead>
<tr>
<th>Solvent</th>
<th>No. Samples</th>
<th>C16 (Phospholipid)</th>
<th>C18 (Triglyceride)</th>
<th>C18:1 (Cholesteryl ester)</th>
<th>C20 (Fatty Acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_3$OH</td>
<td>2</td>
<td>-7.6$^a$</td>
<td>+12.2</td>
<td>-5.9</td>
<td>+1.3</td>
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<tr>
<td></td>
<td></td>
<td>-15.0</td>
<td>+14.3</td>
<td>-12.7</td>
<td>+13.4</td>
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<tr>
<td>CH$_3$OH-CH$_2$Cl$_2$</td>
<td>2</td>
<td>+3.1</td>
<td>+5.3</td>
<td>-7.2</td>
<td>-1.2</td>
</tr>
<tr>
<td>(9:1, v/v)</td>
<td></td>
<td>+2.3</td>
<td>+5.1</td>
<td>-7.7</td>
<td>+0.3</td>
</tr>
<tr>
<td>CH$_3$OH-CH$_2$Cl$_2$</td>
<td>4</td>
<td>-1.4</td>
<td>+0.2</td>
<td>+0.1</td>
<td>+1.1</td>
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<tr>
<td>(6:1, v/v)</td>
<td></td>
<td>-1.0</td>
<td>+1.2</td>
<td>+2.3</td>
<td>-2.5</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>+1.3</td>
<td>+0.8</td>
<td>-2.0</td>
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<tr>
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<td></td>
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</tr>
<tr>
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<td>-0.6</td>
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</tr>
</tbody>
</table>

$^a$Difference in relative per cent (found minus calculated).
TABLE IV

Effect of Gas Quantity on BCl₃ Transesterification of a Standard Lipid Mixture Determined by Recoveries of Fatty Acids.

<table>
<thead>
<tr>
<th>Gas (gms)</th>
<th>#Bubbles (gas)</th>
<th>#Samples</th>
<th>C₁₆ (Phospholipid)</th>
<th>C₁₈ (Triglyceride)</th>
<th>C₁₈:₁ (Cholesteryl ester)</th>
<th>20:0 (Fatty Acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.19-0.30</td>
<td>200-300</td>
<td>3</td>
<td>-5.2  (^a)  ((-6.9 \text{ to } -3.8))</td>
<td>+2.2  ((-0.4 \text{ to } +3.9))</td>
<td>-3.5  ((-2.8 \text{ to } -4.6))</td>
<td>+6.5  ((+3.5 \text{ to } +9.7))</td>
</tr>
<tr>
<td>0.32-0.36</td>
<td>325-350</td>
<td>6</td>
<td>-0.3  ((-1.3 \text{ to } +0.2))</td>
<td>+1.4  ((+0.7 \text{ to } +2.0))</td>
<td>-0.5  ((-1.3 \text{ to } +0.4))</td>
<td>-0.6  ((-1.8 \text{ to } +1.2))</td>
</tr>
<tr>
<td>&gt; 0.39</td>
<td>&gt; 400</td>
<td>3</td>
<td>+4.9  ((+3.5 \text{ to } +7.5))</td>
<td>+10.1 ((+9.6 \text{ to } +11.1))</td>
<td>-18.5 ((-16.8 \text{ to } -22.0))</td>
<td>+3.5  ((+3.4 \text{ to } +3.7))</td>
</tr>
</tbody>
</table>

\(^a\) Mean difference in relative per cent (found minus calculated).

\(^b\) Range of replicate differences.
### TABLE V

**Fatty Acid Composition of Mouse Fibroblasts Following Direct BCl$_3$ Transesterification Under Established Conditions.**

<table>
<thead>
<tr>
<th>Fatty Acid Carbon No.</th>
<th>Sample $^a$</th>
<th>#1</th>
<th>#2</th>
<th>#3</th>
<th>#4</th>
<th>Mean $^b$</th>
<th>#5</th>
<th>#6 $^c$</th>
<th>#7</th>
<th>#8</th>
<th>Mean $^b$</th>
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<tbody>
<tr>
<td>14:0</td>
<td></td>
<td>1.7</td>
<td>1.9</td>
<td>2.2</td>
<td>2.1</td>
<td>2.0</td>
<td>2.1</td>
<td>1.0</td>
<td>1.2</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>15:0</td>
<td></td>
<td>0.2</td>
<td>0.3</td>
<td>0.2</td>
<td>0.3</td>
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<td>0.8</td>
<td>0.7</td>
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<tr>
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<td>2.9</td>
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<td>2.7</td>
<td>2.7</td>
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<td>1.4</td>
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<td>1.0</td>
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<td>1.5</td>
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<td>1.6</td>
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<td>19.2</td>
<td>19.4</td>
<td>19.4</td>
<td>19.3</td>
</tr>
</tbody>
</table>

| mg fatty acid/mg cells | 58.76 | 58.28 | 57.71 | 58.99 | 58.44 | e |

---

*a* Samples 1-4 were aliquots containing 2.00 mg dry weight.

*b* Sample 5 was the methyl ester spot recovered from TLC of pooled aliquots of Samples 3 and 4.

*c* Samples 6-8 were aliquots containing 0.80 mg dry weight from a different cell culture pool.

*d* Relative per cent.

*e* Internal standard omitted.
PART III

RE-EVALUATION OF THE FATTY ACID COMPOSITION OF

CULTURED AORTIC CELLS FROM WHITE CARNEAU

AND SHOW RACER PIGEONS
Aorta cells cultured from atherosclerosis-susceptible White Carneau pigeons were reported deficient in arachidonate when compared with cells from resistant Show Racer cultures (1). Comparisons of biosynthesis, incorporation and transformation of arachidonate between cultures of both breeds were made to determine the nature of the reported deficiency (Koes, M. L., Ph.D. dissertation). The apparent lack of breed differences in the metabolism of arachidonic acid prompted a re-evaluation of the total fatty acid composition of aorta cells cultured from both breeds of pigeons.

Total fatty acids in aorta cells cultured from both sexes of each breed were analyzed as previously described (Koes, M. L., Ph.D. dissertation), and the results are shown in Table I. No significant (P < .05) sex or breed differences were found.

Our inability to demonstrate a deficiency of arachidonic acid in the White Carneau cultures may be due to the use of a modified methanolysis procedure. The original procedure employed by Smith et al (1) may cause losses in polyunsaturated fatty acids due to excess catalyst concentrations (Koes, M. L., Ph.D.)
dissertation). The preferential loss of 20:4w6 from the White Carneau cultures may have resulted from their greater fragility (2) which would promote more rapid cell dissolution and exposure of lipid to boron trichloride. When conditions of methanolysis were modified for small tissue samples by adjustment of catalyst concentration and solvent ratios (Koes, M. L., Ph.D. dissertation) no breed difference in 20:4w6 could be demonstrated.

In addition to a different procedure, Smith et al (1) used embryos produced by birds less than one year old during their first laying season whereas embryos in this study were obtained from birds three or more years old. Young and Middleton (3) have shown that levels of cholesterol arachidonate in serum and liver from eight-month old White Carneau pigeons are significantly lower than eight-month old Show Racers. However, no differences were noted between breeds at five years of age (4). Marcel et al (5) have reported extensive variation in the conversion of 18:2 to 20:4w6 in rat liver microsomal preparations due to age, diet and physiological state of the rats. Therefore, variation in cholesterol arachidonate levels,
which were shown to be breed and age dependent in adult birds (4, 5), may have influenced the arachidonate content in aortas of developing embryos. The nutritional environment in vitro may have a similar effect on cultured cells. Variabilities in the composition of chick embryo extract or horse serum used in culture fluids (McMullin, G. F., unpublished) may also have contributed to the discrepancy in our findings.

Furthermore, the origin of eggs used in both studies was not controlled. It is possible that two metabolically distinct strains of White Carneau were providing embryos for the two studies. Bell et al (6) have shown that individual White Carneau pigeons vary considerably in their response to a cholesterol containing diet. Certain pigeons, termed "hyporesponders", maintained essentially the same serum cholesterol levels despite cholesterol feeding; while others, "hyperresponders", demonstrated significantly higher levels on a cholesterol diet. Cultured cells derived from these two groups could conceivably differ in arachidonic acid levels since polyunsaturated fatty acids have been shown to be instrumental in decreasing plasma cholesterol
levels (7).

The argument as to whether 20:4w6 is, or was, deficient in the White Carneau cells becomes somewhat ambiguous in view of the fact that 22:1w9 and 20:3w3 with respective retention times of 3.71 and 3.73 cannot be distinguished from 20:4w6 on GLC (Koes, M. L., Ph.D. dissertation). Since the total amount of the composite fraction was usually less than one microgram, the lowest limit of linear response of our GLC system, it was impossible to collect that fraction, separate it on TLC, and then re-chromatograph to calculate the quantity of each fatty acid. Such quantitation would be feasible with more sensitive instrumentation such as flame ionization GLC.

It is obvious that refinements in methodology and instrumentation are essential when dealing with small sample sizes. The complex nature of the atherosclerotic process makes it desirable to employ, insofar as possible, a rigidly controlled experimental system. The age of the adult pigeon, its ability to respond to cholesterol overload and the origin of the embryo should be evaluated prior to tissue culture in order to select embryos produced by the "most" resistant and
susceptible birds of the respective breeds. Aorta cells from these strains should permit a better basis for study of the disease process in vitro.
REFERENCES


### TABLE I

Higher Fatty Acid Composition of Cultured Pigeon Aorta Cells

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>White Carneau</th>
<th>Show Racer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male(5) Female(5)</td>
<td>Male(2) Female(5)</td>
</tr>
<tr>
<td>14:0</td>
<td>0.7 b 0.5</td>
<td>0.4 0.7</td>
</tr>
<tr>
<td>15:0</td>
<td>0.5 0.5</td>
<td>0.6 0.5</td>
</tr>
<tr>
<td>16:0</td>
<td>21.1 18.4</td>
<td>19.6 22.1</td>
</tr>
<tr>
<td>16:1</td>
<td>1.0 0.6</td>
<td>1.7 0.8</td>
</tr>
<tr>
<td>18:0</td>
<td>26.9 27.2</td>
<td>26.4 27.2</td>
</tr>
<tr>
<td>18:1</td>
<td>10.7 13.3</td>
<td>12.3 13.0</td>
</tr>
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<td>12.4 15.2</td>
<td>12.8 12.2</td>
</tr>
<tr>
<td>18:3</td>
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<td>1.2 1.0</td>
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<td>1.5 3.0</td>
</tr>
<tr>
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<td>1.9 2.7</td>
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<tr>
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<td>8.2 9.1</td>
<td>8.4 8.2</td>
</tr>
<tr>
<td>22:6</td>
<td>9.1 7.9</td>
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</tr>
<tr>
<td>Total</td>
<td>97.3 98.8</td>
<td>96.6 98.3</td>
</tr>
</tbody>
</table>

Note: Trace amounts of iso 16:0, iso 18:0, 20:1, 20:2, 22:2, and 20:5 were also detected.

a) Numbers in parentheses indicate the number of culture pools analyzed.

b) Relative per cent.
PART IV

PROBLEMS IN THE USE OF $^{14}$C LABELLED POLYUNSATURATED

FATTY ACIDS AS METABOLIC TRACERS
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INTRODUCTION

It is well recognized that certain precautions must be practiced when working with polyunsaturated fatty acids (PUFA) (1). Since these materials autooxidize and polymerize rapidly in air at room temperature, they must be handled in an inert atmosphere and at reduced temperature in so far as possible (2). Samples should also be stored under nitrogen at low temperatures (-20 C) in solvents such as benzene, petroleum ether, chloroform or methylene chloride (2). Storage in alcohol containing solvents must be avoided to prevent formation of methyl esters (3).

Since procedures for isolation and purification of PUFA are both lengthy and involved, the specified chemical and isomeric purity of such acids is always questionable. Some PUFA with certain chain length and number of double bond combinations are poorly resolved by gas-liquid chromatography (GLC), and both argentation thin-layer chromatography (TLC) and GLC are necessary for chemical purification. Confirmation of chain length is most readily accomplished by hydrogenation, followed by GLC of the resulting saturated fatty acids. Since
most PUFA isolated from animal sources have the methylene interrupted type of unsaturation (4), determination of double bond position generally implies elucidation of the W series\textsuperscript{a} either by ozonolysis or oxidation (5). Finally, verification of the geometric (cis-trans) isomer is necessary. This criterion can be established by argentation TLC in which the trans isomers migrate more rapidly than corresponding cis forms (6) or by infrared spectroscopy (IR) in which trans isomers have a characteristic absorption (7). TLC methods must be employed with caution, however, since PUFA left on a TLC plate overnight undergo appreciable isomeric changes (7). Consequently, maintenance of the naturally occurring all cis configuration throughout purification procedures is difficult and may contribute to the fact that some commercially available materials are not entirely as specified (8).

The use of radioactively labelled PUFA may be a source of additional problems. Storage of labelled PUFA of high specific activity gives rise to self-radiation decomposition which can be minimized by adding solid supports or solvents to absorb a portion of the radiation

\textsuperscript{a} Number following the W in subsequent usage denotes position of the first double bond counting from the terminal methyl group.
energies (2). A combination GLC and TLC procedure is the method of choice for establishing radiochemical purity of $^{14}\text{C}$ labelled PUFA (9), but position of the label is difficult to demonstrate without stepwise degradation.

The importance of optimizing GLC operating conditions must not be underestimated in dealing with radioactive materials. Retention of small amounts of radioactivity from thermally altered PUFA and their elution in subsequent analyses may be a source of error. A similar phenomenon has been reported for undegraded radioactive triglycerides (10). Misinterpretation can also result from simultaneous direct and indirect conversion of a radioactive PUFA to products analytically indistinguishable on GLC. Design of such experiments and the interpretation of data require extreme caution.

It is our purpose to describe difficulties which we encountered in studying arachidonate metabolism in pigeon aorta cell cultures using commercially available linoleic acid–U–$^{14}\text{C}$ (18:2–U–$^{14}\text{C}$) and arachidonic acid–1–$^{14}\text{C}$ (20:4–1–$^{14}\text{C}$).
MATERIALS AND METHODS

Reagents

All solvents were reagent grade and redistilled prior to use.

Precautions

All samples were maintained in a nitrogen atmosphere as much as possible during handling and stored under nitrogen at -20 C in methylene chloride or benzene. Temperatures during solvent evaporations never exceeded 25 C.

Thin layer chromatography

Argentation thin layer chromatography was used to separate polyunsaturated fatty acids on the basis of number of double bonds present. Samples were spotted on a silica gel plate impregnated with 5% silver nitrate which had been activated by heating at 110 C for 30 minutes. The chromatography chamber was lined with filter paper and saturated by a 30 minute equilibration period with 250 ml of the developing solvent, diethyl ether: glacial acetic acid, 5000:1 (v/v). Spots were located by viewing under ultraviolet light after spraying with 2', 7'- dichlorofluorescein and identified by comparison with reference standards obtained from Analabs, Inc. and The Hormel Institute. Spots were scraped into glass scintillation vials
for determination of radioactivity in a Packard dual channel liquid scintillation spectrometer. Quench corrections were made by the channels ratio method.

Radioactive compounds retained on the GLC packing were eluted with 100 ml CH₂Cl₂:CH₃OH, 9:1 (v/v), after removal of the packing from the column and concentrated in vacuo. As much as 4% of the total sample was spotted on plates precoated with silica gel G (Analtech) and developed with petroleum ether: diethyl ether: glacial acetic acid, 84:15:1 (v/v). Spots were located by viewing adjacent standards under ultraviolet light after spraying with Rhodamine 6 G. Zones representing the origin, non-esterified fatty acids (NEFA), methyl esters, and solvent front were scraped into glass scintillation vials for determination of radioactivity.

Micro-methanolysis

Pigeon aorta cells incubated with 18:2-U⁻¹⁴C were subjected to BCl₃ micro-methanolysis (Koes, M. L., Ph.D. Thesis).

Gas-liquid chromatography

All gas-liquid chromatographic analyses were performed on a Barber-Colman model 10 chromatograph with
a strontium-90 argon ionization detector. The 6 ft x 0.25 in glass column was packed with ethylene glycol succinate (EGS) (14% on 80/90 mesh Anakrom A) and maintained at 187°C with 15 psi inlet pressure (flow rate - 60 ml/min). Retention times were either directly measured for standards or calculated on the basis of available reference standards (Analabs, Inc. and The Hormel Institute) by the method of Ackman (11).

Radioactive fractions emerging from the GLC system were collected by passing the effluent gas stream from the argon ionization detector through a heated line (235°C) to a 7.5 cm glass cartridge (5 mm O.D.) connected to the GLC exit by a 5 cm teflon tube (1.5 mm O.D.). The cartridge contained silica gel (2.5 cm) into which the teflon tube extended (3 mm) and was plugged at both ends with glass wool. Contents of the cartridge were rinsed with 15 ml "liquifluor" (New England Nuclear Corp.) into a scintillation vial for determination of radioactivity.

RESULTS

Chemical Purity of Arachidonic acid-1-14C

In order to verify the chemical purity of commercially available 20:4-1-14C (Applied Sciences
Laboratories), 50 uc of newly received acid was spotted on silica gel impregnated with 5% silver nitrate (12), and developed with diethyl ether: glacial acetic acid, 5000:1 (v/v). The results obtained are shown in Figure 1.

Use of glacial acetic acid, which is necessary to minimize formation of streaks and trailing of free fatty acids (13), is imperative when dealing with non-esterified polyunsaturated fatty acids. When the volume of glacial acetic acid exceeded 0.25% of our total solvent mixture, free fatty acid spots merged. At a concentration of 1.0% no separation of erucic acid (22:1w9), eicosatrienoic acid (20:3w3), and arachidonic acid (20:4w6) was observed.

Partial identification of the five components in sample A (Fig. 1) was made by comparison with commercially available standards. It should be noted that sample C, 20:3w3, separated into two distinct spots of approximately equal intensity. This acid with a designated purity of 99% may form geometric isomers upon contact with silica gel. The spot closer to the solvent front is probably trans 20:3w3 followed by the cis isomer (6).

Distribution of radioactivity among the five components of sample A is shown in Figure 1. Although no further attempt was made to characterize spots 3 and 4,
it is likely that they represent a mixture of various cis-trans isomeric forms of 20:4\textsubscript{w6}. Since the 20:4-L\textsuperscript{14}C allegedly possessed 65+% all cis isomer (manufacturer's specifications), spot 4 presumably contains the all cis isomer in addition to other cis-trans forms to account for its severe trailing. Spot 3, by virtue of its proximity and slightly greater mobility, may be all trans 20:4\textsubscript{w6} (6) or another contaminant. Spot 5 was not identified, but did contain significant radioactivity.

Metabolism of 18:2-U\textsuperscript{14}C to Products Indistinguishable by GLC

The major anabolic end-product of linoleic acid is arachidonic acid (14) which, in methyl ester form, has a relative retention time of 3.81 on an EGS column (Table I). Under the same operating conditions methyl erucate (22:1\textsubscript{w9}) and methyl eicosatrienoate (20:3\textsubscript{w3}) with retention values of 3.71 and 3.73, respectively, cannot be completely resolved from 20:4\textsubscript{w6} (Table I). We employed argentation TLC in our study of 18:2-U\textsuperscript{14}C metabolism in pigeon aorta cells to separate these three methyl esters and demonstrated the presence of \textsuperscript{14}C in each. Radioactivity in 20:3\textsubscript{w3} and 22:1\textsubscript{w9} was necessarily derived from recycled acetate-U\textsuperscript{14}C, the product of 18:2\textsubscript{w6} degradation,
whereas activity in 20:4w6 most probably resulted from direct conversion.

Other PUFA methyl esters, chromatographed as previously described, also possess nearly identical retention values (Table I). Activity in those esters of the w6 series such as 18:3w6, 20:2w6, 20:3w6 and 20:5w6 (Table I) would be expected to result primarily from direct conversion of 18:2w6 while radioactivity in the w3 and w9 esters must result from degradation of linoleic acid to acetate-U-\(^{14}\)C which is recycled.

Retention of Radioactivity by GLC system

Samples of GLC packing from columns I and II, which had been used respectively for approximately 10 and 60 separations of radioactive methyl esters, ranging from 3,000 to 90,000 dpm per sample, were mixed with "liquid-fluor" for determination of radioactivity. The results are shown in Table II. The majority of radioactivity remained at the origin, and the amount retained increased with column use. However, after repeated injections the radioactivity appeared throughout the column.

Solvent extracts of the GLC packings removed from columns I and II were spotted on TLC. The only discrete spots were located at the origin and front, and
the greatest amount of radioactivity was found at the origin with a lesser amount at the solvent front. Radioactivity contained in these spots, as well as in zones representing free fatty acids and methyl esters, is shown in Table III. It appears that some fatty acid methyl esters as well as free fatty acids are retained on EGS.

An estimation of the interference of GLC system contaminants with manual fraction collection is shown in Table IV. Effluent gas was collected manually at nearly continuous intervals over a 140 minute period following non-radioactive solvent injection. The column had not been used for several hours prior to solvent injection, but the operating flow of argon had been maintained. A single injection of solvent eluted radioactivity at a progressively decreasing rate in the relatively new column, but in the older column levels of radioactivity in the effluent were erratic and considerably higher.

DISCUSSION

Arachidonic acid-$^1_{14}\text{C}$, with a designated radio-purity of 99%, was also stated to be of high chemical purity. Evidence for the chemical homogeniety of this compound was based on argentation TLC (25% silver nitrate)
with two solvent systems: n-hexane: diethyl ether: acetic acid, 89:10:1 (v/v) and chloroform: benzene, 3:2 (v/v) (15).

Our finding of several spots from this highly pure 20:4-1-14C may be attributed to the use of a different solvent system. A satisfactory TLC check for purity of PUFA should include at least two solvent systems: a less polar one, such as n-hexane: diethyl ether, 4/1 (v/v) to distinguish saturated, mono-, and dienoic acids and a more polar system, such as diethyl ether: glacial acetic acid, 5000:1 (v/v) or diethyl ether: methanol, 9:1 (v/v), to separate tri-, tetra-, penta-, and hexaenoic acids (16).

A combination of GLC, TLC, and IR should be employed to adequately establish chemical and isomeric purity. Some investigators have relied solely on GLC for determination of the chemical purity of 20:4-1-14C (17) and 20:3w6-U-14C (18). Since other PUFA have retention times similar to those of 20:4w6 and 20:3w6 on both EGS (Table I) and ethylene glycol adipic acid columns (11) additional criteria for purity are imperative. Likewise, the importance of establishing isomeric purity of a chemically pure PUFA must not be underestimated since
Beare-Rogers and Ackman (8) report the presence of considerable quantities of trans isomers in commercially available PUFA, and such was found to be the case in the present study.

The partial overlapping of methyl esters on GLC may also give rise to ambiguity, particularly in continuous flow systems where the GLC response is coupled to the radioactivity response of a scintillation spectrometer. Argentation TLC represents a more sensitive tool for the separation and further identification of unsaturated methyl esters especially from small tissue samples.

Radioactivity in acids of the w6 series (Table I), such as 20:4w6, should arise primarily from direct conversion of 18:2-U-14C and result in products of relatively high specific activity. Labelled 14C in the w3 and w9 acids listed in Table I, such as 20:3w3 and 22:1w9, must originate indirectly by incorporation of acetate-14C. These acids should possess lower specific activities by comparison with products converted directly from a uniformly labelled acid. Although not reported here, such data is essential to unequivocally establish metabolic phenomena. Studies of 18:2-1-14C and 20:4-1-14C metabolism in rat testes also revealed that metabolic breakdown
of these acids resulted in the recycling of acetate-1-$^{14}$C into PUFA (17,19) and palmitic acid. Since 18:2w6 is reported to be rapidly oxidized (20), the extent of acetate recycling must not be overlooked in studies of essential fatty acid metabolism.

Interpretation of GLC data is also confounded by the retention of radioactivity in the GLC system. The irregular distribution of radioactivity that we noted in our GLC packing has also been reported for columns used in the radiochromatography of triglycerides (10). All triglycerides are initially retained on the packing to about the same extent but those of high molecular weight are partitioned more slowly thus explaining their peculiar distribution. In all systems tested Breckenridge and Kuksis (10) reported that recovered radioactivity was due to undegraded triglycerides. The packing contamination which we observe is probably due in part to retention of fatty acid methyl esters as well as free fatty acids, a result of incomplete esterification. However, examination of Table III reveals that the greatest amount of radioactivity is found at the origin of the TLC plate with a lesser amount at the front. These fractions may result
from the formation of thermal polymers and breakdown products or they may represent lipids such as phospholipids or cholesterol esters which were not transesterified. This latter possibility cannot be excluded since it is difficult to achieve complete methanolysis particularly in small samples (Koes, M. L., Ph.D. Thesis). Attempts to achieve complete methanolysis would require increased catalyst concentrations and/or increased reaction times which invariably result in losses of PUFA (21).

Column packing contamination is not the only source of radioactive interference. A major problem in the use of fraction collection and particularly in continuous counting is radioactive trailing caused by partial retention of components in tubes leading to the fraction collector or to the radioactivity detector. This possibility must be examined with great care especially if the sequential collection of low levels of radioactivity is employed. In continuous flow systems it is recommended that the liquid scintillator be drained or the solid scintillator tube replaced to restore background to a low level (22). In such systems it is also wise to reduce the length of all transfer lines to minimize
trailing (10). While our system possessed well-heated, short connections the level of contaminant radioactivity emerging in the effluent gas was still considerable, particularly after prolonged use (Table IV). An estimation of the actual amounts of radioactivity contributed by retention on the GLC packing and by condensation in delivery tubes respectively was not made, although the comparison of Tables III and IV suggests that retention of label on the column followed by slow elution was the major contributing factor.

Experimental systems in which fractions of low radioactivity must be quantitated are subject to error upon radiochromatography unless actual sample activity can be distinguished from GLC system contamination. Under these circumstances it would be advisable to determine both the amount of contamination prior to sample injection and the radioactivity eluted by solvent injection during the intervals corresponding to periods of fraction collection. Breckenridge and Kuksis (10) also recommend allowing extra time between samples for venting of trailing activity through the detector at maximum column temperature to avoid contamination of subsequent samples. However, each sample injection appears to cause additional
elutions of radioactivity. Furthermore, these authors emphasize the importance of optimizing the uniformity of GLC packing, the thickness of the liquid phase coating and the flow rate of carrier gas to insure best recovery of triglycerides. Without doubt, thorough consideration of all of these factors is necessary, at least to minimize radioactive trailing on GLC.
ACKNOWLEDGEMENTS

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REFERENCES


TABLE I

Similarity of GLC Retention Times of Certain Polyunsaturated Fatty Acids

<table>
<thead>
<tr>
<th>Acid</th>
<th>Retention Time a</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:3w6</td>
<td>1.92 *</td>
</tr>
<tr>
<td>18:3w3</td>
<td>2.02 '</td>
</tr>
<tr>
<td>20:2w9</td>
<td>2.71 '</td>
</tr>
<tr>
<td>20:2w6</td>
<td>2.74 *</td>
</tr>
<tr>
<td>20:3w9</td>
<td>3.36 '</td>
</tr>
<tr>
<td>20:3w6</td>
<td>3.40 '</td>
</tr>
<tr>
<td>22:1w9</td>
<td>3.71 *</td>
</tr>
<tr>
<td>20:3w3</td>
<td>3.73 *</td>
</tr>
<tr>
<td>20:4w6</td>
<td>3.81 *</td>
</tr>
<tr>
<td>20:4w3</td>
<td>4.61 '</td>
</tr>
<tr>
<td>20:5w6</td>
<td>4.72 '</td>
</tr>
</tbody>
</table>

a Retention times were either directly measured (*) from standards or calculated ('') on the basis of available reference standards (Analabs, Inc. and The Hormel Institute) by the method of Ackman (12). Analyses were performed on a column packed with ethylene glycol succinate (14% on 80/90 mesh Anakrom A) and maintained at 187°C with 15 psi inlet pressure (flow rate - 60 ml/min).
### TABLE II

**Distribution of Radioactivity Retained on Ethylene Glycol Succinate Packing of GLC Columns**

<table>
<thead>
<tr>
<th>Section of Column</th>
<th>Column I</th>
<th>Column II</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Inches from Inlet)</td>
<td>dpm/100 mg packing</td>
<td></td>
</tr>
<tr>
<td>0-2</td>
<td>102</td>
<td>564</td>
</tr>
<tr>
<td>17-19</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>35-37</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>53-55</td>
<td>0</td>
<td>41</td>
</tr>
<tr>
<td>69-71</td>
<td>0</td>
<td>22</td>
</tr>
</tbody>
</table>

---

**a** Portions of the EGS packing from sections indicated obtained after collecting data in Table IV were weighed and mixed with liquifluor for determination of radioactivity.

**b** Received approximately 10 injections ranging from 3,000 to 30,000 dpm per injection.

**c** Received approximately 60 injections ranging from 3,000 to 90,000 dpm per injection.
TABLE III
Radioactivity in Lipid Classes Extracted\textsuperscript{a} from Spent GLC Packings

<table>
<thead>
<tr>
<th>TLC fraction</th>
<th>Column I Total dpm\textsuperscript{b}</th>
<th>Column II Total dpm\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Origin</td>
<td>1415</td>
<td>4219</td>
</tr>
<tr>
<td>Free Fatty Acid</td>
<td>200</td>
<td>3034</td>
</tr>
<tr>
<td>Methyl Ester</td>
<td>68</td>
<td>1533</td>
</tr>
<tr>
<td>Front</td>
<td>104</td>
<td>1430</td>
</tr>
<tr>
<td>Total</td>
<td>1787</td>
<td>10216</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Radioactive compounds retained on GLC packing of Columns I and II remaining after collecting data in Table II were eluted with 100 ml methylene chloride: methanol, 9/1, (v/v) and concentrated in vacuo. As much as 4\% of the total sample was spotted on plates precoated with silica gel G (Analtech) and developed with petroleum ether: diethyl ether: glacial acetic acid, 84:15:1, (v/v). Spots were located by viewing adjacent standards under ultraviolet light after spraying with Rhodamine 6G.

\textsuperscript{b} Individual spots were scraped into glass scintillation vials for determination of radioactivity. Activities in the aliquot spotted were corrected to give the total dpm of each fraction retained on the GLC column.
TABLE IV

Contaminant Radioactivity in Effluent Gas from GLC Columns

<table>
<thead>
<tr>
<th>Collection Interval</th>
<th>dpm eluted/minute</th>
<th>( \text{Column I} )</th>
<th>( \text{Column II} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-15</td>
<td>7.2</td>
<td>57.2</td>
<td></td>
</tr>
<tr>
<td>15-20</td>
<td>5.2</td>
<td>96.2</td>
<td></td>
</tr>
<tr>
<td>20-40</td>
<td>4.2</td>
<td>43.2</td>
<td></td>
</tr>
<tr>
<td>40-50</td>
<td>3.3</td>
<td>37.7</td>
<td></td>
</tr>
<tr>
<td>50-110</td>
<td>2.5</td>
<td>19.4</td>
<td></td>
</tr>
<tr>
<td>110-140</td>
<td>1.3</td>
<td>20.8</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>440</strong></td>
<td><strong>4373</strong></td>
<td></td>
</tr>
</tbody>
</table>

\( a \) Represents time period during which effluent was collected following non-radioactive solvent injection.

\( b \) Had previously received approximately 10 injections ranging from 3,000 to 30,000 dpm per injection.

\( c \) Had previously received approximately 60 injections ranging from 3,000 to 90,000 dpm per injection.
<table>
<thead>
<tr>
<th>Spot No.</th>
<th>% of Total Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Trace</td>
</tr>
<tr>
<td>2</td>
<td>0.2</td>
</tr>
<tr>
<td>3</td>
<td>18.7</td>
</tr>
<tr>
<td>4</td>
<td>56.8</td>
</tr>
<tr>
<td>5</td>
<td>24.3</td>
</tr>
</tbody>
</table>

Fig. 1. Tracing of thin layer chromatogram on silica gel impregnated with 5% silver nitrate developed with diethyl ether: glacial acetic acid (250 ml:0.05 ml). Spots were visualized with 0.2% 2'7'-dichlorofluorescein in ethanol. A represents 50 uc 20:4-1\(^{14}\)C (Sp. Act. 52mc/mM) (Applied Science Laboratories); B, C and D represent non-radioactive reference standards, 20:4w6, 20:3w3 and 22:1w9, respectively (Analabs, Inc.).
BIOGRAPHICAL DATA

Name in Full       Mary Lavoie Koes
Date of Birth     December 15, 1942
Place of Birth    Nashua, New Hampshire
Secondary Education Mount Saint Mary Seminary
                                 Nashua, New Hampshire

Collegiate Institutions attended Dates Degrees
Rivier College       1960-1964         B.A.
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                                   Delta Epsilon Sigma
                                   Phi Sigma
                                   Sigma Xi

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University of New Hampshire Graduate school teaching assistantship 1964-1966
Research project assistant, University of New Hampshire 1966-1967
Assistant Scientist, Cancer Research Center, Columbia, Missouri 1970-