4. Vitamin $K_1$ suspended in the same way as α-tocopherol was almost equally effective as a reactivating agent.

5. The addition of α-tocopherol did not affect the degree of inhibition of the DPNH oxidase system by antimycin.

6. No evidence was obtained that α-tocopherol would be oxidized by oxygen or by cytochrome *c*, or that the quinone could be reduced by DPNH, in the presence of the heart-muscle preparation.

7. It is suggested that the inactivation by iso-octane extraction is caused by adsorption of iso-octane on the surface of the enzyme or by some physical damage of the particle of the enzyme preparation, and that α-tocopherol reactivates by dissolving the iso-octane or by restoring the physical structure of the particle. The experiments do not provide strong evidence for the participation of α-tocopherol in the respiratory chain, nor do they disprove it.

## REFERENCES


1 J. BOUMAN AND E. C. SLATER, *Biochim. Biophys. Acta*, 26 (1957) 624.
2 A. NASON AND I. R. LEHMAN, *Science*, 122 (1955) 19.
3 A. NASON AND I. R. LEHMAN, *J. Biol. Chem.*, 222 (1956) 511.
4 A. NASON, B. C. AVERBACH AND A. J. TERRELL, *Biochim. Biophys. Acta*, 19 (1956) 395.
5 E. C. SLATER, J. BOUMAN AND L. VELDSTRA, *Acta Physiol. Pharmacol. Neerl.*, 5 (1956) 209.
6 D. KEILIN AND E. F. HARTREE, *Biochem. J.*, 41 (1947) 500.
7 E. C. SLATER, *Biochem. J.*, 45 (1949) 1.
8 P. KARRER AND A. GEIGER, *Helv. Chim. Acta*, 23 (1940) 4550.
9 A. EMMERIE AND C. ENGEL, *Rec. Trav. Chim.*, 57 (1938) 1351.
10 E. C. SLATER, *Biochem. J.*, 46 (1950) 484.
11 L. SMITH, in *Methods in Enzymology*, ed. by S. P. COLOWICK AND N. O. KAPLAN, Vol. 2, p. 732, Academic Press, New York, 1955.
12 J. P. COLPA-BOONSTRA AND E. C. SLATER, *Biochim. Biophys. Acta*, 23 (1957) 222.
13 J. BOUMAN AND E. C. SLATER, *Nature*, 177 (1956) 1181.
14 K. O. DONALDSON AND A. NASON, *Proc. Natl. Acad. Sci. U.S.*, 43 (1957) 364.
15 O. WARBURG, *Schwermetalle als Wirkungsgruppen von Fermenten*, Verlag Dr. Werner Saenger, Berlin, 1948.


Received August 17th, 1957

# INCORPORATION OF RADIOACETATE INTO LIPID BY ADIPOSE TISSUE *IN VITRO*: LIPID CHARACTERIZATION

J. P. MILLER* AND JOHN A. D. COOPER

*Department of Biochemistry, Northwestern University Medical School, Chicago, Ill. (U.S.A.)*

## INTRODUCTION

The ability of adipose tissue to incorporate glucose or acetate into lipid has been established by several investigators[1–5]. This investigation is concerned with characterization of the lipid components synthesized by adipose tissue from $^{14}$C-1-acetate in an *in vitro* system.

## METHODS

### *Lipid separation*

Lipid separation was accomplished by a modification of the chromatographic and extraction procedure described by BORGSTROM[6]. The column used consisted of silicic acid** (13 g) which had

* Present address: Nutrition Department, Abbott Laboratories, North Chicago, Ill.

** Mallinckrodt Chemical Works, Silicic Acid-100 mesh (suitable for chromatographic analysis by the method of Ramsey and Patterson).

*References p. 144.*

been activated by heating at 118°C for 18 hours. The silicic acid was mixed with one-half its weight Hyflo Super-Cel, slurried in Skellysolve B and packed (under nitrogen pressure) in a 2.5 cm jacketed* column to a height of 13 cm. The column was subsequently washed with the following solvents** *seriatim:* benzene, chloroform and Skellysolve B.

The column was charged with up to 150 mg mixed lipid in 10 ml Skellysolve B and rinsed onto the column with two more 10 ml aliquots of Skellysolve B. Elution was then carried out as follows: (1) Cholesterol ester was eluted from the column with 1:9 benzene in Skellysolve B followed by 2:8 and finally 3:7 benzene–Skellysolve B. (2) Triglyceride, fatty acid and cholesterol were then eluted together with benzene followed by chloroform. (3) Phospholipid was eluted from the column with methanol.

The column was allowed to flow by gravity at a flow rate of 0.5 ml per minute. Lipid components were identified by qualitative tests (Lieberman-Burchard for cholesterol and cholesterol ester and phosphorous test for phospholipid) and by examination on a monolayer film[7]. This latter procedure was standardized in this laboratory by Dr. S. H. MAI and consists of placing a drop of the lipid in question (dissolved in petroleum ether) on a film of oil*** which has been spread on a water surface. This method not only indicates the presence or absence of the lipid component but also shows the type of lipid component present. On the monolayer film cholesterol ester appears as a white residue, glycerides as oil drops or expanding figures, cholesterol and fatty acids as rapidly expanding clear figures and phospholipids as fast creeping figures, respectively.

Following elution, separation of the middle fraction (containing triglyceride, fatty acid and cholesterol) was accomplished by a modification of BORGSTROM's[6] extraction procedure. This procedure consisted of first separating fatty acid from triglyceride and cholesterol by extraction with ethanolic sodium hydroxide. The triglyceride-cholesterol fraction was then saponified and the triglyceride fatty acid separated from the cholesterol by again extracting with ethanolic sodium hydroxide.

Typical recovery results of two known lipid mixtures by this chromatographic and extraction procedure are given in Table I. The mixtures contained radiocarbon-labelled fatty acids to permit quantitation of this component by radioisotopic as well as gravimetric methods.

TABLE I

RECOVERY OF KNOWN LIPID MIXTURES IN SEPARATION PROCEDURE

| *Component* | *Expt.* | *Mg added* | *Percent recovery (by weight)* | *Radioactivity in fraction percent* |
|---|---|---|---|---|
| Cholesterol-stearate | (1) | 12.03 | 94.3 | None |
| | (2) | 10.87 | 96.5 | 0.1 |
| Tripalmitin | (1) | 23.52 | 103.2 | 0.7 |
| | (2) | 36.05 | 101.5 | 0.3 |
| Cholesterol | (1) | 24.83 | 98.0 | 0.2 |
| | (2) | 22.11 | 94.0 | 0.1 |
| Labelled fatty acid* | (1) | 25.72 | 107.8 | ← 93.9 |
| | (2) | 25.72 | 99.4 | ← 99.5 |
| Lecithin** | (1) | 22.40 | 94.5 | — |
| | (2) | 9.41 | 108.3 | ← 0.9 |

* Purified fatty acid mixture obtained from *in vitro* incubations (specific activity—2040 c.p.m./mg).

** Prepared according to the method of PANGBORN[8].

*Removal of acetate contamination*

Before separation of lipid components by the previously described method it is necessary to remove any traces of contaminating $^{14}$C-acetate from the lipid extract of the *in vitro* incubation mixture. This was accomplished by a modification of a procedure described by FOLCH *et al.*[9].

* Column was kept at a temperature of 25°C by water circulation.

** All solvents were reagent grade and were dried and distilled before use.

*** Cenco Hyvac Oil heated in a thin layer at 175°C for 8–10 hours to a surface tension of 17 dynes/cm$^2$.

*References p. 144.*

The efficiency of this method was proved as follows: Three ml incubation medium, 258 mg minced adipose tissue and 100 $\mu$l (5$\mu$c) $^{14}$C-1-acetate were thoroughly homogenized with a Teflon homogenizer in 57 ml (19:1) of 2:1 chloroform–methanol. This homogenate was then filtered through Whatman No. 42 filter paper. The filtrate was washed with one-third volume water followed by two one-third volume aliquots of 0.05% $CaCl_2$. These washes (upper aqueous–methanol phase) were combined and subsequently extracted four times with 5 ml aliquots of Skellysolve B. The Skellysolve B extract and original chloroform (lower phase) were combined yielding the lipid extract which contained only 0.008% of the original radioactivity from $^{14}$C-1-acetate.

To determine whether this procedure caused a loss of lipid the same procedure was carried out using a labelled fatty acid mixture instead of $^{14}$C-1-acetate. The final lipid extract was found to contain 94.1% of the radioactivity from the labelled fatty acid while the wash contained only 0.9%.

*Incubation and counting procedure*

Minced rat epididymal adipose tissue plus 3 ml medium and 100 $\mu$l $^{14}$C-1-acetate was incubated for 3 at 37.5 °C in a stoppered flask. All samples were counted in a scintillation counter* incorporating a coincidence circuit and a two-channel pulse height analyzer.

## RESULTS AND DISCUSSION

Normal rat epididymal adipose tissue (246 mg) was incubated with 3 ml of a tissue culture medium (Table II) and 5 $\mu$c of $^{14}$C-1-acetate. Following incubation this mixture was homogenized (2:1 chloroform–methanol), washed to remove contaminating $^{14}$C-acetate, and the lipid extract separated into individual components according to the methods previously described.

TABLE II

TISSUE CULTURE MEDIUM §

*I. Amino acids* (L *only*)

| | | | | | |
|---|---|---|---|---|---|
| Aspartic | 0.8 | Serine | 12.0 | Phenylalanine | 9.2 |
| Asparagine | 6.0 | Threonine | 15.2 | Histidine:HCl | 14.0 |
| Glutamic | 8.0 | Cysteine:HCl | 5.2 | Ornithine:HCl | 10.0 |
| Glycine | 16.0 | Methionine | 4.0 | Arginine:HCl | 18.0 |
| Alanine | 34.0 | Taurine | 6.0 | Lysine:HCl | 32.0 |
| Valine | 30.0 | Proline | 25.0 | Cystine | 8.0 |
| Leucine | 17.2 | Tyrosine | 11.2 | Glutamine | 92.0 |
| Isoleucine | 10.0 | Tryptophan | 12.0 | | |

*II. Vitamins*

| | | | |
|---|---|---|---|
| Biotin | 0.04 | *p*-Aminobenzoic acid | 0.08 |
| Folic acid | 0.08 | Choline chloride | 12.0 |
| Ca pantothenate | 0.50 | Inositol | 6.0 |
| Pyridoxine:HCl | 0.25 | Ascorbic acid | 14.0 |
| Thiamine | 0.16 | $\alpha$-Tocopherol $PO_4$ | 2.0 |
| Riboflavin | 0.64 | Nicotinamide | 1.0 |

*III. Inorganic*—EARLE'S *salt solution*[10]

*IV. Glucose*—1000.0

§ Kindly supplied by Dr. FLOYD C. MCINTIRE, Abbott Laboratories, North Chicago, Ill. All concentrations above are given in mg/l.

It should be noted that during the chromatography of the lipid extract from the *in vitro* system an appreciable amount of oily material was eluted from the column with 3:7 benzene–Skellysolve B (nothing was eluted with 1:9 or 2:8 benzene–Skelly-

* Tri-Carb Counter, Model 314, Packard Instrument Co., LaGrange, Ill.

*References p. 144.*

solve B). This material did not contain any cholesterol ester as determined by Lieberman-Burchard reaction and monolayer film studies but consisted of triglyceride. In establishing the method with known lipid mixtures it was found that nearly all of the cholesterol ester was eluted with 2:8 benzene–Skellysolve B and the remainder eluted with 3:7. Triglyceride was not eluted until much higher proportions of benzene were attained. This discrepancy is probably due to the fact that the lipid extract from the *in vitro* system contained appreciable amounts of unsaturated triglyceride as evidenced by its oily nature while the original column was developed using a saturated triglyceride (*i.e.* tripalmitin).

TABLE III

CHARACTERIZATION OF LIPID SYNTHESIZED BY ADIPOSE TISSUE *in vitro*

| *Component* | *Percent (by weight)* | *Percent (by radioactivity)* | *Percent of total acetate incorporated/100 mg tissue* |
|---|---|---|---|
| Lipid extract | 100.0 | 100.0 | 1.99 |
| Cholesterol ester | None | None | None |
| Triglyceride | 97.9 | 92.0 | 1.83 |
| Fatty acid | 0.46 | 1.23 | 0.02 |
| Cholesterol | 0.31 | 0.14 | 0.003 |
| Phospholipid | 0.53 | 2.03 | 0.04 |

Results of this experience and characterization of the *in vitro* synthesized lipid components are given in Table III. It is obvious that the lipid synthesized by adipose tissue consists almost entirely of triglyceride in this system. The amounts of other components formed are negligible and indeed may represent contamination from the triglyceride fraction instead of actual synthesis.

## SUMMARY

Normal minced rat epididymal adipose tissue incubated *in vitro* with $^{14}$C-1-acetate incorporates the label almost entirely into the fatty acids of triglycerides with negligible incorporation into other lipid components (including cholesterol esters, cholesterol, non-esterived fatty acid, and phospholipid).

## REFERENCES

[1] D. D. FELLER, *J. Biol. Chem.*, 206 (1954) 171.
[2] P. FAVARGER AND J. GERLACH, *Helv. Physiol. et Pharmacol. Acta*, 13 (1955) 96.
[3] F. X. HAUSBERGER AND S. W. MILSTEIN, *J. Biol. Chem.*, 214 (1955) 483.
[4] G. ROSE AND B. SHAPIRO, *Biochim. Biophys. Acta*, 18 (1955) 504.
[5] J. P. MILLER AND J. A. D. COOPER, *Biochim. Biophys. Acta*, 24 (1957) 430.
[6] B. BORGSTROM, *Acta Physiol. Scand.*, 25 (1952) 111.
[7] K. K. JONES, *Quart. Bull. Northwestern Univ. Med. School*, 24 (1950) 253.
[8] M. C. PANGBORN, *J. Biol. Chem.*, 188 (1951) 471.
[9] J. FOLCH, M. LEES, AND G. H. SLOANE-STANLEY, *Federation Proc.*, 13 (1954) 209.
[10] W. R. EARLE, E. L. SCHILLING, T. H. STARK, N. P. STRAUS, M. F. BROWN AND E. SHELTON, *J. Natl. Cancer Inst.*, 4 (1943) 165.

Received July 11th, 1957