4. Zeller, E. A., Barsky, J., *ibid.*, 1952, v81, 459.

5. Dasler, W., Millister, R. W., *ibid.*, 1958, v98, 759.

6. Ponsetti, F. V., Wawzonek, S., Shepard, R. S., Evans, T. C., Stearno, G., *ibid.*, 1956, v92, 366.

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## Comparative Metabolic Studies of $H^3$ and $C^{14}$ Labeled Stearic Acid.* (26041)

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With the introduction of liquid scintillation spectrometers, beta-emitting tracers began appearing more frequently in studies using radioisotopes. Tritiated compounds have been thought advantageous because of ease in preparation, high specific activity, low cost, and relative safety. However, we have often considered whether $H^3$ might also be fraught with properties that do not lend it to accurate tracing of specifically labeled fatty acids. With these thoughts in mind, the following investigation was undertaken to compare metabolism of $H^3$ and $C^{14}$ labeled stearic acid.

*Materials and method. Tissue absorption studies.* Approximately 0.6 mg of stearic-1-$C^{14}$ and 0.15 mg of stearic-9, 10-$H^3$ (5-10 $\mu$c) were mixed in 1 cc of corn oil and fed by means of stomach tube into male Sprague-Dawley rats weighing 300-450 g. They were maintained on a diet of Purina Fox Chow before and during experiments. Animals were decapitated at intervals of 15, 30, 45, 60, 90, 120, 180, and 240 minutes. Livers and epididymal fat pads were excised and homogenized in methanol or immediately frozen at −15°C. Tissue lipids were extracted with chloroform-methanol as described by Van Handel and Zilversmit(1). Five milliliter aliquots of the extracts were then evaporated and placed in counting solvent, consisting of 0.4% PPO† and 0.01% POPOP‡ in reagent grade toluene. Counts were made in a Packard Tri-Carb Liquid Scintillation C-14 Spectrometer. Simultaneous equations were then used to determine the separate values contributed by each of the 2 labels when both were counted simultaneously. Counting at HVT 5, window 10-80, efficiencies of around 50% for $C^{14}$ and 0.3% for $H^3$ were obtained; while at HVT 9, window 10-50, efficiencies of around 11% for $C^{14}$ and 15% for $H^3$ were obtained. Thirty-two animals were studied in this manner. *Lipid fraction studies.* In these analyses the dose was raised to 50 $\mu$c per type of stearic acid so as to increase sample specific activity. Aliquots analyzed by the silicic acid columns were reduced to 0.0125 cc for fat and 0.025 cc for liver compared to the 5 cc aliquots used in the absorption studies. Animals were decapitated 4 hours post injection, liver and fat pad were weighed, then immediately placed in the homogenizer. Chloroform-methanol extracts were further processed to remove protein contamination by the method suggested by Enteman(2), using a flash evaporator at 60°C, successive chloroform-methanol extractions, and filtration through fat-free filter paper. The lipid obtained was then fractionated on silicic acid columns according to the method of Hanahan (3). To check the manner in which differently labeled fatty acids were handled by the silicic acid columns, we put known concentrations of stearic-1-$C^{14}$ and stearic-9, 10-$H^3$ on the columns and then carried out the usual elution procedure.

*Results. Liver and epididymal fat pad absorption.* Variation when comparing $H^3$ to $C^{14}$ in specific activity of the labels in each tissue is apparent. Variations of similar magnitude were found in the counts of the same type labeled stearate in different animals killed at the same time (Table I). On some

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† PPO or 2, 5-diphenyloxazole.

‡ POPOP or p-bis (2-(5-phenyloxazole))-benzene from Pilot Chemicals, Inc., Watertown, Mass.

TABLE I. $H^3$ and $C^{14}$ Stearic Acid Absorption Studies.*

| Min. | % $H^3$ dose/g tissue, mean & stand. dev. | | % $C^{14}$ dose/g tissue, mean & stand. dev. | |
|---|---|---|---|---|
| Fat pad | % | | | |
| 15 | .818 | .80 | 1.09 | 1.27 |
| 30 | 2.25 | 2.32 | 2.18 | 1.47 |
| 45 | 9.0 | 1.65 | 7.2 | 3.88 |
| 60 | 10.9 | 6.82 | 13.5 | 11.9 |
| 90 | 38.4 | 14.3 | 46.8 | 33.2 |
| 120 | 26.0 | 23.5 | 36.8 | 29.2 |
| 180 | 105.5 | 75.3 | 114.8 | 90.7 |
| 240 | 94.1 | 61.4 | 114.0 | 83.0 |
| Liver | | | | |
| 15 | 2.0 | .61 | 1.5 | .23 |
| 30 | 6.1 | 3.24 | 7.6 | 4.4 |
| 45 | 29.7 | 8.8 | 27.8 | 11.9 |
| 60 | 58.4 | 27.6 | 43.9 | 20.6 |
| 90 | 198.1 | 157.0 | 134.4 | 66.3 |
| 120 | 192.6 | 335.0 | 172.4 | 123.0 |
| 180 | 365.2 | 232.0 | 320.0 | 197.6 |
| 240 | 405.8 | 107.6 | 410.9 | 254.0 |

* Each measurement made in 4 animals.

occasions $C^{14}$ deposition seemed greater than $H^3$. But all these mentioned variations were not consistent nor statistically significant ($p>0.5$). Uptake of each label in the tissues examined seemed most rapid in 1½ hours. Also, specific activity of liver lipid was greater than that of the adipose tissue.

*Lipid fractionation studies.* The 4 fractions separated by silicic acid chromatography were: (a) cholesterol esters, (b) triglycerides and free fatty acids, (c) free cholesterol, monoglycerides, diglycerides, (d) phospholipids. Results in this part of the investigation showed a tendency for $C^{14}$ and $H^3$ stearic acids in the fat pads to go to fraction b, less to fraction c, and a negligible amount to fraction d. In liver the labels were found to be concentrated in fraction d with a small but significant amount in fractions b and c (Table II). More $H^3$ was incorporated into the lipid fractions with the exception of liver phospholipid.

Table III summarizes our findings when stock $C^{14}$ and $H^{13}$ stearic acid were run through the silicic acid column. This points up a consistent and fairly sizable contamination of the tritiated fatty acid into the free cholesterol, monoglyceride and diglyceride fraction, and a lesser contamination in the cholesterol ester fraction. No such contamination was found in the case of $C^{14}$.

*Discussion.* Since its introduction, tritium has seemed attractive as a label for use in tracer studies. It has been used widely in industry with apparent success and more recently in many biochemical studies(4). However, the question arises as to whether $H^3$ would stick firmly to a fatty acid vehicle—not be liberated and/or exchanged freely with non-radioactive H + during any significant chemical manipulation. It was hoped that this study would help elucidate some of the tracer properties of $H^3$ for fatty acids. In looking for a vehicle fatty acid we decided upon stearic acid in that the work of Glascock and Reinius(6) indicated the $H^3$-stearic bond was stable. Our absorption studies in-

TABLE III. Recovery Studies.

| Fraction | Stearic-1-$C^{14}$ | Stearic-9, 10-$H^3$ |
|---|---|---|
| | % activity | |
| a | .38 | 1.41 |
| b | 97.30 | 89.60 |
| c | 1.78 | 8.55 |
| d | .54 | .49 |

TABLE II. Tissue Lipid Fractionation Studies. % administered dose/100 g tissue.

| | $H^3$ | | | | | $C^{14}$ | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Exp. | | | | | Exp. | | | | |
| Fat pad | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 |
| | (%) | | | | | | | | | |
| a | .0 | .8 | .0 | .0 | .0 | .0 | 1.2 | .0 | .0 | .0 |
| b | 4.3 | 5.9 | 7.1 | 12.0 | 5.7 | 4.4 | 7.1 | 10.6 | 17.9 | 4.7 |
| c | .55 | | .81 | 1.1 | 1.2 | .65 | | 9.4 | 1.9 | .32 |
| d | .21 | .23 | .01 | 1.2 | .8 | 1.1 | 1.9 | 2.0 | 1.6 | 2.2 |
| Liver | | | | | | | | | | |
| a | .15 | .51 | .18 | .17 | .27 | .16 | .9 | .45 | .56 | .38 |
| b | 2.3 | 7.8 | 5.7 | 5.3 | 4.4 | 3.8 | 6.5 | 8.1 | 9.4 | 6.5 |
| c | .28 | .75 | .93 | .65 | .69 | .42 | .84 | 1.4 | 1.5 | .66 |
| d | 13.8 | 33.0 | 29.0 | 28.0 | 31.0 | 12.0 | 22.8 | 32 | 28 | 15.0 |

dicate no G. I. alteration of the $H^3$-stearic acid compared to $C^{14}$-stearic acid.

During chloroform-methanol lipid extraction, the possibility was considered that $H^3$ might exchange with $H_2O$ washings. This possibility was checked by testing portions of the non-lipid water-methanol fractions for radioactivity; none was found. From this it would appear that the label deposited in both liver and epididymal fat pad remained in the lipid as extracted.

Due to previously demonstrated aberrations in phospholipid fractions during fatty acid studies(1,7) in which a fatty acid molecule was altered by a label, the remainder of this work dealt with comparisons of $H^3$ and $C^{14}$ within the lipid fractions as separated by silicic acid columns. Variation was most evident in the fat pad, especially for both fraction c and fraction d. Finding the label in fraction c, especially for the fat pad, was of interest. It has been shown that fat tissue produces little cholesterol(8,9). This then represented incorporation of the labels into monoglycerides and diglycerides, deposition of liver cholesterol in the fat pad, or was of some significance in generation of cholesterol. Because of this finding plus the fact that $H^3$ seemed to show this phenomena more than $C^{14}$, we considered the possibility that this resulted from alterations to the $H^3$ label brought on by the silicic acid columns. 8.6% of the stock $H^3$ labeled stearic acid turned up in fraction c (Table III) when processed through the column. Here, then, was the first glaring example of apparent inaccuracy in use of the $H^3$ labeled stearic acid. Even accounting for $H^3$ stearic acid contamination, the count in fraction c is impressive. According to the studies in rat liver of Dittmer and Hanahan(3) using stearic-1-$C^{14}$, stearic acid has a propensity to incorporate into both phospholipid and esterified cholesterol. What we observed, then, may be an extension of this phenomenon to fat tissue, may represent some form of cholesterol transport, or may indicate incorporation of $H^3$ into monoglycerides and diglycerides. Further investigation already under way seems to indicate the last possibility.

*Summary.* The stability of orally administered stearic-9, 10-$H^3$ appears comparable to that of stearic-1-$C^{14}$ in its incorporation into lipid in rat liver and epididymal fat pad. However, column fractionation of tissue lipids and stock labeled fatty acids suggests a difference in behavior of stearic acid labeled in these 2 ways.

1. Van Handel, E., Zilversmit, D. B., *J. Lab. and Clin. Med.*, 1958, v52, 831.

2. Colowick, S., Kaplan, N., *Methods in Enzymology III*, 1955, Academic Press, 310.

3. Dittmer, J. C., Hanahan, O. J., *J. Biol. Chem.*, 1959, v234, 1976.

4. *Proceedings of Symposium on Advances in Tracer Applications of Tritium, N. Y.*, Boston, Sheldon Press, 1958.

5. Blomstrand, R., Dahlback, O., Linder, E., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 768.

6. Glascock, R. T., Reinius, L. R., *Biochem. J.*, 1956, v62, 529.

7. Artom, C., Peretti, G., *Boll. Soc. Ital. Biol. Sper.*, 1932, v7, 930.

8. Feller, D., *J. Biol. Chem.*, 1954, v206, 171.

9. Miller, J. P., Cooper, J. A., *Biochem. Biophys. Acta,*

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## Growth of Feline Viral Rhinotracheitis Virus in Cultures of Feline Renal Cells. (26042)

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The isolation of a virus which induces rhinotracheitis in cats and produces a cytopathic change in cultures of feline renal cells has been reported(1,2). This report deals with the growth of feline viral rhinotracheitis (FVR) virus in cultures of feline renal cells and the