Elongation of [ω - ^{14}C]Oleic Acid and [ω - ^{14}C]Nervonic Acid

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During feeding experiments with [ω - ^{14}C]oleic acid and [ω - ^{14}C]nervonic acid to adult rats, ^{14}C -labelled C_{26} , C_{28} and C_{30} fatty acids were recovered from the intestinal mucosa, liver, plasma, kidney and stools. The structures of these fatty acids were determined by g.l.c., radio-g.l.c. and mass spectrometry. The Schmidt and Ginger degradation methods indicated that most of the ^{14}C found in these extra-long fatty acids remained in the ω position. These radioactive extra-long fatty acids were found mainly in the polar lipids of rats killed 3 or 15 h after being fed on labelled oleic acid or nervonic acid. Rats killed 63 h later yielded only traces of these extra-long fatty acids. When the rats were given antibiotics or received the same radioactive fatty acids by intravenous injection, the labelled extra-long fatty acids could not be detected in any of the tissues. We conclude that they were probably synthesized by elongation of oleic acid and nervonic acid by intestinal micro-organisms (probably yeasts) and then absorbed by the intestinal mucosa.

During experiments involving the addition of trace quantities of [ω - ^{14}C]oleate and [ω - ^{14}C]nervonate to the diet of adult male Wistar rats, it was found that the polar lipid fractions of the intestinal mucosa, liver, plasma, kidney and stools contained ^{14}C -labelled extra-long fatty acids (C_{26} – C_{30}). The object of this study was to determine their origin.

Materials and Methods

Radioactive compounds

[24- ^{14}C]Nervonic acid (specific radioactivity 0.131 mCi/mg) and [18- ^{14}C]oleic acid (specific radioactivity 0.177 mCi/mg) were purchased from C.E.A., Gif-sur-Yvette, France. The radiochemical purity of these compounds as determined by radio-g.l.c. was $\geq 99.9\%$.

Animals

Male adult Wistar rats (400 g) were fed with individual doses of 200 μCi of [24- ^{14}C]nervonic acid or 200 μCi of [18- ^{14}C]oleic acid as described by Chevallier & Lutton (1966). Groups of rats were killed at 3, 15 and 63 h after the ingestion of the radioactive diet. The tissues of each of these rats were removed, washed, weighed and samples were taken for analysis.

Chemical methods

Samples of faeces and of individual tissues were extracted with chloroform/methanol (2:1, v/v) (Folch *et al.*, 1957). After being washed and dried,

one sample of the extract was subjected to silicic acid (Bio-Sil A, 100–200 mesh; Bio-Rad Laboratories, Richmond, CA, U.S.A.) column chromatography to separate neutral lipids (by chloroform elution) from phospholipids (by methanol elution) (Marsh & Weinstein, 1966). Individual lipid families were obtained from the separated neutral lipids and phospholipids by chromatography on thin-layer plates and each was individually identified and assayed (Rabinowitz *et al.*, 1971). Each of the lipid families was hydrolysed for 2 h with 10% (w/v) KOH, and the isolated fatty acids were converted into methyl esters (Morrison & Smith, 1964).

G.l.c. was performed by using a Packard model 846 with a 6 ft-long glass column packed with SE-30, silicone gum rubber or diethylene glycol succinate (all from Supelco, Bellefonte, PA, U.S.A.). Argon was used as the carrier gas. Temperature programming was carried out from 160° to 200°C at 1°C per min; the column was then kept at this temperature for 45 min more. The retention time and mass spectrometry were used for the identification of the fatty acids. Area measurements were used for quantification. Authentic fatty acid methyl esters in known amounts assayed under precisely the same conditions were used as standards.

Radio-g.l.c. To establish which fatty acids in a mixture were radioactive, the exit gas-line of the Packard gas-chromatography apparatus, provided with a non-destructive argon ionization mass detector, was coupled through a heated connector to a Popjak integrating liquid scintillator (Rabinowitz & Hercker, 1974) or to a Packard 894 proportional counter (Chase & Rabinowitz, 1967).

Chemical oxidative degradation of fatty acids. The determination of the radioactivity of the carboxyl carbons and the alkyl carbons of fatty acids was determined by use of the Schmidt method (Rabinowitz, 1968). Mild oxidation (Ginger, 1944) was used for the isolation of the terminal two-carbon group, and the recovered acetic acid was then subjected to the Schmidt degradation method. By means of these techniques the ω -methyl group was separated, then oxidized to CO_2 . The $^{14}\text{CO}_2$ from the above methods was collected (Rabinowitz, 1968) in a 5% solution of Hyamine hydroxide in toluene, and assayed for radioactive content on a Tri-Carb liquid-scintillation counter. Counting was performed to a $\pm 3\%$ standard deviation (Chase & Rabinowitz, 1967).

Mass spectrometry. This was made available by courtesy of Dr. J. Zarembo of the Smith Kline and French Labs. (Philadelphia, PA, U.S.A.). A Perkin-Elmer Hitachi RMU-6-E electron-impact magnetic-focusing medium-resolution 1-in-4000 mass spectrometer was used. Samples were analysed by using a direct insertion probe at 70 eV at 100°C at 0.13 mPa. The methods for fatty acids (McCloskey, 1970) were used in the presentation and interpretation of data.

Micro-hydrogenation. This was performed by the addition of 100 mg of 5% Pt/charcoal catalyst in 20 ml of ether to 10–15 mg of the unsaturated fatty acids. H_2 gas was bubbled through the mixture. After hydrogenation was completed (25–30 min), as judged by spot tests for unsaturation (e.g. by using Br_2/water or KMnO_4), the catalyst was filtered off and the solution evaporated to dryness (Rabinowitz, 1968).

Results and Discussion

Adult male rats were killed 3, 15 or 63 h after being fed on a meal containing ^{14}C -labelled fatty acid. Intestinal mucosa, liver, plasma, kidney and stools from rats 3 and 15 h after being fed on $[\omega\text{-}^{14}\text{C}]\text{joleic}$ acid or $[\omega\text{-}^{14}\text{C}]\text{nervonic}$ acid yielded several large radioactive fatty acid peaks on radio-g.l.c.; the corresponding mass peaks were very small. The retention times of these peaks suggested that they were materials of high molecular weight. Reported retention times of very-long-chain fatty acids suggested that these peaks corresponded to C_{26} , C_{28} and C_{30} fatty acid methyl esters. Authentic samples of these methyl esters were obtained from Analtech, Newark, DE, U.S.A. The retention times observed for them were very close to, but not identical with, the values for the unknown materials. Since the authentic samples were saturated fatty acids, the possibility existed that the unknown materials were unsaturated. By the use of pooled samples and preparative g.l.c., sizeable amounts of these unknown materials were obtained. These samples were then

subjected to mild continuous micro-hydrogenation (Rabinowitz, 1968). Approx. 80–85% of the H_2 necessary for saturation of one double bond was utilized in each case. The results suggested that perhaps one double bond may be present in these fatty acids. The recovered hydrogenated derivatives of the various unknown peaks were rechromatographed under the same conditions. This time the observed retention times were identical with those of the authentic samples. Samples of the fully hydrogenated and methylated materials from the unknown peaks were also analysed by mass spectrometry (McCloskey, 1970). The results obtained for the unknown samples and for the methylated authentic C_{26} , C_{28} and C_{30} fatty acids were indistinguishable from each other. The structures of these fatty acids were reconfirmed by the use of two methods (mass spectrometry and g.l.c.), new samples obtained from tissues of various rats being used each time.

Only in samples obtained from polar fractions (phosphatidylcholine, phosphatidylethanolamine and sphingomyelin) of some tissues was there sufficient radioactivity in the extra-long fatty acid fraction for assay. The neutral-lipid fractions only showed traces of these materials regardless of the tissues examined. Six separate experiments were carried out to determine what fraction of the total radioactivity appeared in extra-long-chain fatty acids. The results were averaged, and the standard error of the mean was calculated (Table 1). The values obtained are the percentage of the radioactivity recovered in extra-long fatty acids on an individual-tissue basis. (The recovered radioactivity in the fatty acid fraction of each tissue was considered to be 100%.) Rats fed on $[\omega\text{-}^{14}\text{C}]\text{joleate}$ had approximately the same percentage of extra-long fatty acids in their tissues as did those fed on $[\omega\text{-}^{14}\text{C}]\text{nervonate}$, both at 3 and 15 h. The tissue with the highest percentage of these extra-long fatty acids was the intestinal mucosa. The proportions of the C_{26} , C_{28} and C_{30} fatty acids varied with different rats; in most tissues the C_{26} and C_{28} chains predominated. When tissue samples were examined 63 h after feeding of the labelled precursor, there were only trace amounts of radioactivity in both the polar and neutral lipids.

To determine whether these extra-long fatty acids were produced by chain elongation (Whereat *et al.*, 1967; Rabinowitz, 1968; Rabinowitz & Hercker, 1974), the samples were subjected to the Schmidt and Ginger degradation procedures. The results indicated that most (>90%) of the total ^{14}C label of each sample was in the alkyl fraction, and the ω -carbon itself contained 55–65% of the label. Less than 10% was found in the carboxyl carbon.

This elongation may have been carried out by the rat or by micro-organisms in the intestinal tract. To test this, rats were treated with antibiotics

Table 1. *Percentage of total radioactivity recovered in the extra-long fatty acid fraction*

The results are the means $\pm$ s.e.m. of six experiments (each with four rats). Trace indicates that there was approximately 0.3% of the total radioactivity.

	% of total radioactivity recovered						
	Stools	Intestinal mucosa	Liver	Plasma	Kidney	Adipose pad	Lung Muscle
Time after fed with [ω - 14 C]oleate (h)							
3	12 $\pm$ 4	23 $\pm$ 7	2 $\pm$ 1	2 $\pm$ 2	2 $\pm$ 1	—	—
15	15 $\pm$ 6	10 $\pm$ 3	6 $\pm$ 2	8 $\pm$ 2	4 $\pm$ 2	Trace	—
63	—	Trace	—	—	—	Trace	—
Time after fed with [ω - 14 C]nervonate (h)							
3	13 $\pm$ 4	27 $\pm$ 3	2 $\pm$ 2	3 $\pm$ 1	2 $\pm$ 2	—	—
15	17 $\pm$ 3	14 $\pm$ 3	7 $\pm$ 2	5 $\pm$ 1	2 $\pm$ 1	Trace	—
63	—	Trace	—	—	Trace	Trace	—

[penicillin (25000 i.u.) or tetracycline (50 mg added to 25000 i.u. of nystatin)]. These animals did not yield 14 C-labelled extra-long fatty acids from their tissues when fed on labelled oleate or nervonate. Intravenous injections of the ω - 14 C-labelled fatty acids into animals treated with antibiotics also failed to yield the 14 C-labelled extra-long fatty acids. A literature search revealed that some *Saccharomyces cerevisiae* strains may contain these extra-long fatty acids in their mitochondria (Welch & Burlingame, 1973). Microbiological cultures of the stools and intestinal mucosa of all of these animals revealed the presence of yeast. This yeast, when grown with either [ω - 14 C]oleate or [ω - 14 C]nervonate by the usual techniques (Welch & Burlingame, 1973; Tochikura *et al.*, 1970), yielded a mixture of these extra-long fatty acids; a total of $18 \pm 3\%$ of the fatty acid radioactivity recovered in this fraction was in these extra-long fatty acids. (The chemical yield was less than 1%.)

The yeast present in the rat intestine could be responsible for the elongation of the [ω - 14 C]oleic acid and [ω - 14 C]nervonic acid. The newly formed extra-long unsaturated fatty acids (C_{26} , C_{28} and C_{30}) in the yeast mitochondria could be digested in the rat lumen and then absorbed by the intestinal mucosa. From the mucosa these lipids could be distributed via the plasma to various tissues (e.g. liver, kidney), where they could be metabolized. The small content (traces) of labelled extra-long fatty acids in tissues of rats killed 63 h after feeding of the 14 C-labelled precursor may indicate a metabolic turnover or translocation of these fatty acids to depot fat.

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References

- Chase, G. & Rabinowitz, J. L. (1967) *Radioisotope Methodology*, 3rd edn., pp. 285–287
- Chevallier, F. & Lutton, C. (1966) *Bull. Soc. Chim. Biol.* **48**, 507–514
- Folch, J., Lees, M. & Sloane-Stanley, G. N. (1957) *J. Biol. Chem.* **226**, 497–511
- Ginger, L. G. (1944) *J. Biol. Chem.* **156**, 453–456
- Marsh, J. B. & Weinstein, D. B. (1966) *J. Lipid Res.* **7**, 574–576
- McCloskey, J. A. (1970) in *Topics in Lipid Chemistry* (Gunston, F. D., ed.), vol. 1, pp. 337–469, Logos Press, London
- Morrison, W. R. & Smith, L. M. (1964) *J. Lipid Res.* **5**, 600–608
- Rabinowitz, J. L. (1968) *Anal. Biochem.* **22**, 498–510
- Rabinowitz, J. L. & Hercker, E. S. (1974) *Arch. Biochem. Biophys.* **161**, 621–627
- Rabinowitz, J. L., Bailey, T. A. & Marsh, J. B. (1971) *Arch. Oral Biol.* **16**, 1195–1205
- Tochikura, T., Kimura, A., Tachiki, T. & Gotan, T. (1970) *J. Ferment. Technol.* **48**, 763–768
- Welch, J. W. & Burlingame, A. L. (1973) *J. Bacteriol.* **115**, 464–466
- Whereat, A. F., Hull, F. E., Orishimo, M. W. & Rabinowitz, J. L. (1967) *J. Biol. Chem.* **242**, 4013–4022