Biosynthesis of ^{14}C -labeled Linoleic Acid and Its Fate in Normal and Fat-deficient Rats

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The effects of linoleic acid and of other "essential" fatty acids as curatives for the fat-deficiency syndrome have been rather extensively studied, but far less data are available concerning absorption, distribution, and intermediary metabolism of these compounds. Attempts have been made to study these problems by means of certain easily demonstrable derivatives of the "essential" acids, such as conjugated fatty acids, but whether these are physiologically equivalent to the original compounds seems rather doubtful. Certainly, a more promising method would be the use of isotope-labeled "essential" fatty acids, but as far as we are aware no reports of such work have up to now been published.

In this paper we describe the biosynthetic production of ^{14}C -labeled linoleic acid and report observations on its absorption, distribution, and metabolism after administration to a normal and a fat-deficient rat.

The biosynthetic incorporation of ^{14}C from carbon dioxide into linoleic acid was accomplished in two steps: (1) the photosynthesis of ^{14}C -labeled sugars by green leaves and (2) the production of ^{14}C -labeled linoleic acid from these sugars by means of a yeast-like organism.

EXPERIMENTAL

Radioactivity determinations. All samples were counted with a thin mica end-window Geiger-Müller counter. Two different mounting techniques were used. Barium carbonate mounts were prepared according to a method essentially identical with that described by Henriques, Kistiakowsky, Margnetti and Schneider¹. Lipids and other organic materials were mounted using a modification of the method of Entenman, Lerner, Chaikoff and Dauben². To facilitate the measurement of samples of low activity it was desirable to get more material mounted than a lens paper alone could hold. Therefore we used aluminum dishes (surface: 19.6 cm²) with 2 mm edges in which the lens paper was placed. As a rule 10 000 counts were collected, but samples with low activity were not counted for more than 32 minutes. All counts were corrected for coincidence, background, self-absorption, and variations in geometry. In the case of lipids and other organic materials, empiric correction factors were used to convert the obtained counts into corresponding

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barium carbonate counts. The statistical evaluation of the counting data was done by means of the generally used formulas based on the Poisson relation.

Photosynthesis of ^{14}C -labeled sugars. The photosynthesis was carried out by means of *Canna indica* leaves according to the method of Hassid ³. As the sugars in the alcoholic leaf extract were to be used only as a substrate in the following microbial synthesis, no efforts were made to isolate them in pure form. The extract was, however, converted into an aqueous solution by gently heating it until all alcohol had evaporated and by simultaneously adding water to keep the volume constant.

Three runs of photosynthesis were carried out with 260 to 400 microcuries of $^{14}\text{CO}_2$ in each *. The total amount of $^{14}\text{CO}_2$ used was 990 microcuries corresponding to 34 million counts per minute under our counting conditions. The utilization of carbon dioxide by the leaf was practically quantitative, and the total yield of radioactivity in the alcoholic leaf extract was 31 million counts per minute or 91 % of the amount given to the leaves.

Microbial synthesis of linoleic acid. The organism used for the production of linoleic acid was *Trichosporon pullulans*, a yeast-like fungus belonging to the group *Mycotoruloidae* **. In earlier work by one of us ⁴ this organism had given good yields of lipids, nearly half of which consisted of linoleic acid.

The yeast was grown as a surface culture in a 1 000-ml Roux flask. The volume of the medium was 300 ml, and its composition was as follows: glucose 4 %, diammonium hydrogen phosphate 0.3 %, $\text{KH}_2/\text{K}_2\text{HPO}_4$ to give the concentration 0.3 M and the initial pH 6.0, and inorganic salts to give the following quantities per liter of the medium: magnesium sulfate 400 mg, sodium chloride 160 mg, calcium chloride 80 mg, ferrous sulfate 40 mg, manganous sulfate 40 mg, and zinc sulfate 16 mg. Aeration was started immediately after inoculation and carried on at a rate of 400 ml per hour.

The sugar consumption of the yeast was followed during the experiment by determining the reducing substances in the medium every other day according to the method of Somogyi ⁵.

To avoid the loss of ^{14}C in the metabolism of the yeast during the period of the most intensive growth, the extract containing the labeled sugars was not added to the culture until on the fifth day, when the sugar concentration of the medium had fallen to about one-half of the initial. The added extract contained about 300 mg of sugars, and its total radioactivity was 29 million counts per minute. Traps containing 2.4 N sodium hydroxide for carbon dioxide collection were connected to the culture flask immediately after addition of the radioactive material. On the tenth day, when about 5 % of the initial amount of sugar remained in the medium, the experiment was stopped.

The yeast mycelium was separated from the medium, transferred into a 250 ml flask, and hydrolyzed with boiling 5 N hydrochloric acid during 20 minutes. The mixture was kept under nitrogen during this process and during further manipulations. The lipids were extracted from the hydrolysate with three 250 ml portions of ether. The combined ether extracts were evaporated, and 4 ml of inactive ethyl linoleate were added as a carrier to the separated lipids. The lipids were saponified with 15 ml boiling 1.8 N ethanolic potassium hydroxide for one hour. The ethanol was evaporated, and the residue dissolved in water. The unsaponifiable material was removed with three successive 50 ml portions of ether. The fatty acids were then liberated by adding 5 ml concentrated hydrochloric acid and extracted with three (50—25—25 ml) portions of low-boiling petroleum ether. The petroleum ether solution was washed with 10 % sodium chloride until neutrality and dried with anhydrous sodium sulfate.

The fatty acids dissolved in petroleum ether were brominated by adding bromine drop by drop under constant stirring at a temperature below -13°C . The insoluble bromination product was filtered on a small Buchner filter and washed with cold petroleum ether. At this stage it weighed 1 350 mg. After recrystallization from ethylene dichloride, the obtained snow-white crystals melted at 115.7°C (corrected). The bromine content of 13.3 mg of the crystals determined according to the method of Willard and Thompson ⁶ was found to be 52.5 % (calculated for tetrabromostearic acid 53.3 %).

* The carbon 14 used in this investigation was supplied by the Oak Ridge National Laboratory, Oak Ridge, Tenn., on authorization from the Isotopes Division, U.S. Atomic Energy Commission.

** This species was supplied by the "Centraalbureau voor Schimmelcultures", Baarn, Netherlands.

Fig. 1. Excretion of ^{14}C activity in the form of carbon dioxide by a normal and a fat-deficient rat after administration of ^{14}C -labeled ethyl linoleate.

920 mg of the purified tetrabromostearic acid was debrominated with zinc and hydrogen chloride in absolute ethanol. The crude ethyl linoleate was purified by distillation under vacuum (2.5–6 mm Hg). The final yield of the purified ester was 403 mg and its specific activity was 232 counts per minute per mg of carbon, corresponding to 73 100 counts per minute for the total sample.

Fate of ^{14}C -labeled linoleic acid in the normal and the fat-deficient rat. The radioactive ethyl linoleate was fed to two female rats, one of which had been maintained on our stock diet and the other on a fat-deficient diet* until it had plateaued in weight. At the beginning of the experiment the normal rat weighed 191 g and the fat-deficient rat 163 g. The age of the former was 146 days and of the latter 183 days.

The ^{14}C -labeled ethyl linoleate was given with a carefully calibrated syringe into the pharynx of the animals. The dose per rat was 176 mg corresponding to 31 800 counts per minute or to about 0.9 microcuries. Each of the animals was then immediately placed into a small metabolism apparatus, which was connected with sodium hydroxide traps for the collection of expired carbon dioxide. Carbon dioxide-free air was drawn through the apparatus at a rate of 6–7 liters per hour.

The animals were kept in the metabolism apparatus for six hours and then killed with ether. The following parts were separated from the carcass: liver, kidneys, heart, brain, subcutaneous tissue, perirenal adipose tissue, mesentery and omentum, genital adipose tissue, and gastrointestinal tract with its contents combined with the excreted feces. These parts were thoroughly homogenized by means of a scalpel, scissors and finally an all-glass homogenizer of the Potter-Elvehjem type. The rest of the carcass was also homogenized, and an aliquot was treated in the same way as the parts mentioned above. The lipids were separated from the homogenized samples by extracting them three or four times with boiling alcohol and thereafter with ether essentially according to the

* Sucrose 72 %, casein, fat-low, 24 %, salt mixture 4 %. Synthetic vitamins were incorporated in the diet to give the following quantities per kilogram of the diet: thiamine 6 mg, riboflavin 8 mg, pyridoxin 4 mg, calcium pantothenate 20 mg, nicotinic acid 20 mg, inositol 500 mg, folic acid 0.3 mg. Vitamin A and alpha-tocopherol were given twice weekly in amounts corresponding to daily doses of 140 I.U. vitamin A and 1 mg alpha-tocopherol per rat.

Table 1. Recovery of ^{14}C activity in the tissues and excretions of a normal and a fat-deficient rat six hours after administration of ^{14}C -labeled ethyl linoleate.

	Normal rat		Fat-deficient rat	
Total recovered activity, % of the administered	89.6		79.6	
Activity remaining in the gastrointestinal tract and its contents, % of the administered	65.4		46.5	
	Recovered activity, % of the absorbed *	Specific activity of lipids, c.p.m./mg	Recovered activity, % of the absorbed *	Specific activity of lipids, c.p.m./mg
Carcass				
Lipids				
Organ				
Liver	10.3	2.4	26.7	7.3
Kidneys	0.5	0.8	1.4	1.7
Heart	0.5	0.8	1.0	3.4
Brain	(0.2) **	(0.1) **	0.4	0.3
Depot				
Subcutaneous	12.9	0.3	4.9	0.2
Perirenal	(0.3) **	(0.02) **	1.3	0.2
Mesenterial and omental	2.6	0.3	5.7	0.6
Genital	(0.9) **	(0.03) **	(1.8) **	(0.1) **
The rest of the carcass	62.8	0.7	45.2	0.8
Non-lipid material	traces	traces	traces	traces
Excretions				
Expired CO_2	7.8	—	9.4	—
Urine	1.2	—	2.2	—
	100.0		100.0	

* Absorbed = total recovered activity minus the activity recovered in the gastro-intestinal tract and its contents.

** These figures represent low counts which do not significantly differ from the background ($P > 0.05$).

methods described by Bloor⁷. Finally the extracted lipids were transferred into petroleum ether and mounted for radioactivity determinations. The lipid-free tissues remaining after extraction and the small alcohol-extracted non-lipid fractions were also tested for radioactivity. The urine was mounted in the same way as the lipids and counted.

The excretion of ^{14}C -labeled carbon dioxide by the two animals during the experiment is shown in Fig. 1. The total excretion of carbon dioxide, indicating the general rate of metabolism, was 24.1 mg CO_2 per hour in the normal rat and 36.4 mg CO_2 per hour in the fat-deficient animal.

The distribution of the ^{14}C -activity at the end of the experiment is given in Table 1.

DISCUSSION

In view of the well-known difficulties in the synthesis of linoleic acid by chemical methods, the biosynthetic preparation of this compound seems to be the only practical procedure available at present. But even the latter

method is not without difficulties. The yields obtained by us cannot be regarded as very satisfactory. The total yield of ^{14}C in the final ethyl linoleate was only about 0.2 % of the amount in the carbon dioxide used as the initial ^{14}C source. The first phase of the biosynthesis could be carried out quite effectively, as over 90 % of the initial radioactivity was recovered in the alcoholic leaf extract. This accords well with many earlier reports on the efficiency of photosynthetic procedures^{8,9}. On the other hand, considerable losses occurred in the second phase. These were partly unexpected as calculations based on previous information of the linoleic acid production by *Trichosporon pullulans*⁴ and also on a number of preliminary experiments with inactive sugar indicated that about 1 % of the initial ^{14}C of sugar should be recoverable in the form of purified linoleic acid. The unduly low yield of linoleic acid may have been caused by an excessively strong alkali treatment to which the lipids were inadvertently subjected during the evaporation of alcohol from the saponification mixture.

The small yield of radioactive ethyl linoleate, unfortunately, permitted animal experiments on a very limited scale only. Therefore, the conclusions based on them are necessarily of a preliminary character. The rate of intestinal absorption of ethyl linoleate was greater in the fat-deficient rat than in the normal one (53 % of the dose given in the former *versus* 35 % in the latter). Thus the need of the body for linoleic acid seems to be one of the factors determining the rate of its absorption. As is known, a similar principle governs the absorption of certain other nutrients, *e.g.* iron^{10,11}.

Less than one-tenth of ^{14}C of the absorbed ethyl linoleate was excreted by the animals as carbon dioxide during the six-hour experiment. The excretion was a little faster in the fat-deficient rat. On the other hand, the general metabolic rate of this animal was considerably higher than that of the normal rat.

At the end of the experiment only traces of radioactivity were found outside the lipid fractions of the carcass. The distribution pattern of the ^{14}C of linoleate showed distinct differences in the two animals. In the fat-deficient rat a proportionally much greater part of the absorbed radioactivity was deposited in the liver (26.7 % *versus* 10.3 % in the normal rat). A similar difference between the two animals was also found in regard to the kidneys and the heart. In the normal rat, on the other hand, the subcutaneous adipose tissue took up a larger percentage of the absorbed radioactivity (12.9 % *versus* 4.9 % in the fat-deficient rat), and such was also the case with the "rest of the carcass" consisting mainly of the muscles and the skeleton (62.8 % *versus* 45.2 % in the fat-deficient rat). These differences seem rather plausible and are obviously explainable by assuming the existence of a vital need for essential fatty acids in organs such as liver, kidneys, and heart. The great avidity with which these organs in the fat-starved animal take up the ^{14}C of linoleate is also reflected by the high specific activities attained by their lipids. Especially the activity of the heart lipids is notable as being over four times that of the normal rat.

In view of the generally known role of the liver in fat metabolism, it is hardly unexpected that a considerable portion of radioactivity would be found retained in this organ — at least a relatively short time after the administration. It is perhaps of more interest to note the high uptake of ^{14}C of

linoleate by the lipids of the heart and of the kidneys, a fact which may be indicative of some specific function of the essential fatty acids in the physiology of these organs.

SUMMARY

^{14}C -labeled linoleic acid was produced biosynthetically in two steps: (1) the photosynthesis of ^{14}C -labeled sugars by *Canna indica* leaves and (2) the synthesis of linoleic acid from these sugars by means of the yeast *Trichosporon pullulans*.

The radioactive linoleic acid was administered in the form of ethyl linoleate to one fat-deficient and one normal rat, and the animals were sacrificed after six hours. In the fat-deficient rat the intestinal absorption of linoleate was more rapid, and a proportionally greater part of the absorbed activity was deposited in the liver, the kidneys, and the heart. In the normal rat the subcutaneous adipose tissue took up relatively more of the absorbed ^{14}C . Less than one-tenth of the activity was excreted as carbon dioxide, and no marked difference was found between the animals in this respect.

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