Lipogenesis from glucose-2-^{14}C and acetate-1-^{14}C in aorta

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ABSTRACT Lipogenesis was measured with glucose-2-^{14}C and acetate-1-^{14}C in the everted aortas of normal and atherosclerotic rabbits. More glucose-2-^{14}C than acetate-1-^{14}C was incorporated into lipids in both the normal and the atherosclerotic aorta.

Radiocarbon from glucose-2-^{14}C appeared mainly in triglycerides and phospholipids with a small amount in cholesteryl esters. Incorporation increased almost threefold with atherosclerosis, most of the radioactivity being in the glycerol moiety; radioactivity was predominantly in carbon 2 of glycerol.

About 70% of the acetate-1-^{14}C incorporated into phospholipids and triglycerides was in the fatty acids, and the remainder was in glyceride-glycerol; 98% of the radioactivity in cholesteryl esters was in the fatty acid moiety. Incorporation into cholesteryl esters was increased most during the development of atherosclerosis.

Fatty acid synthesis was similar from both acetate-1-^{14}C and the 2 carbon unit derived from glucose-2-^{14}C, viz., predominantly de novo synthesis of fatty acids with 14 and 16 carbon atoms, and elongation for those of 18 carbons and longer.

SUPPLEMENTARY KEY WORDS aortic lipogenesis · lipid degradation

Lipogenesis[1] has been studied with various radioactive substrates in normal and atherosclerotic aortas. Several investigators have used glucose-^{14}C to study lipogenesis in intact aortic tissue preparations of various species (1–7). Glucose-^{14}C incorporation into fatty acids (1), phospholipids (4), and total lipids (2, 3, 5, 7) has been measured, and the total lipids have been separated into the individual lipid classes (5). The high degree of incorporation of ^{14}C from labeled glucose into the glycerol fraction of total glycerides compared with the low amount of label in fatty acids has also been noted (1, 4, 6).

In this paper I report studies on lipogenesis in normal and atherosclerotic rabbit aorta with glucose-2-^{14}C and with acetate-1-^{14}C. Data on the incorporation of these two substrates into total lipids, into different lipid classes, and particularly into the glycerol vs. fatty acid moieties of the lipids are compared. Since acetate-1-^{14}C arises from glucose-2-^{14}C the mode of synthesis of the fatty acids can be assessed and compared with results obtained when acetate-1-^{14}C is used as substrate.

MATERIALS AND METHODS

Animals, Diets, and Aorta Preparation

Female New Zealand rabbits were maintained 3–4 months on Purina rabbit chow pellets. The control group received pellets coated with corn oil (Mazola); this diet contained 1.4 g of oil/kg. The atherogenic group received pellets coated with corn oil (1.4 g/kg of diet) containing cholesterol (8) (U.S.P., Nutritional Biochemicals Co.) at a level of 0.8 g/kg of diet.

The rabbits were killed by cervical dislocation; the aorta from the descending arch to the diaphragm was removed and flushed with 0.9% saline solution, and the adherent material was stripped away. The

[1] "Lipogenesis" is defined here as incorporation of radioactive substrate into any portion of the total lipids, viz., glycerol, fatty acids, cholesterol, choline, etc., by any enzymic mechanism. "Fatty acid synthesis" is used to indicate radioactive substrate incorporation only into fatty acids, either by elongation or by de novo synthesis.

Abbreviations: TLC, thin-layer chromatography; PL, phospholipid; TG, triglyceride; CE, cholesteryl ester.

aorta was everted over a glass rod and tied, and the ends were wrapped with Parafilm to exclude access of the medium to the adventitial portion. The aorta was placed in 2.5 ml of Krebs-Ringer salts medium with either 2.8 mM glucose-2-^{14}C (5.4 μCi/μmole) or 2.8 mM acetate-1-^{14}C (10.4 μCi/μmole) (New England Nuclear, Inc., Boston). During the incubation at 39°C for 3 hr, oxygen–carbon dioxide (95%:5%) was bubbled from the bottom of the tube; this ensured adequate movement of the medium over the entire surface.

Analyses

After incubation, the aorta was cut from the glass rod and inspected for the extent of atherosclerosis. The control groups had intimal surfaces that were smooth and lesion free; the rabbits maintained on cholesterol had raised, white lesions which covered 70–95% of the intimal surface. The weights of the intima and most of the media (stripped away from the adventitia) averaged 168 mg and 542 mg for control and atherosclerotic aortas, respectively. The intima plus media was weighed and homogenized in $CHCl_3$–CH_3OH 2:1, and the lipid was extracted (9). The lipid extract was passed through a Sephadex G-25 column (10) to further reduce the amount of nonlipid material. Lipid was separated into classes on TLC according to the method of Skipski et al. (11) with heptane–ethyl ether–acetic acid–methanol 85:15:3.5:2.5. Areas that contained lipids were visualized after the plates were sprayed with 2,6-dichlorofluorescein; the silica gel was scraped into pipets, the lipid was eluted, and aliquots of the eluate were assayed for radioactivity.

To determine if there was any contamination of the lipids by radioactive substrate, nonincubated tissue was homogenized in chloroform–methanol, substrate was added, and the lipids were isolated. The results indicated that no more than 1.4% of the radioactivity in the phospholipids could have been due to contamination from glucose-2-^{14}C and no more than 2.6% from acetate-1-^{14}C. Triglycerides would not have been contaminated by radioactive substrate. When a sufficient amount of radioactivity was present, the lipid was hydrolyzed in ethanolic KOH, excess ethanol was evaporated with nitrogen, water was added, and the aqueous solution was extracted with petroleum ether (bp 40–60°C) before and after acidification of the aqueous portion. Aliquots from the aqueous and petroleum ether fractions were assayed for radioactivity. Under these conditions, the aqueous fraction contains glycerol or glycerophosphate, the petroleum ether extract of the alkaline aqueous layer contains nonsaponifiable compounds, and the petroleum ether extract of the acidified aqueous layer contains the fatty acids.

Substrate Characterization

After the aortas had been incubated with glucose-2-^{14}C, the water-wash of the chloroform-extracted aorta was analyzed to determine the distribution of ^{14}C in acetate. The acetate was isolated, converted to phenylacetophenone, and degraded to iodoform and phenylbenzoic acid according to the procedure of D'Adamo and D'Adamo (12). The distilled acetate-^{14}C was diluted with carrier sodium acetate, converted to acetyl-^{14}C chloride with phosphorus oxychloride (13), and the acetyl-^{14}C chloride was distilled off. Phenylacetophenone-^{14}C was prepared by refluxing the acetyl-^{14}C chloride, biphenyl, and 10% molar excess of anhydrous aluminum chloride in carbon disulfide. Portions of the phenylacetophenone-^{14}C, mp 119–120°C (unc; Ref. 12, 120–121°C), were cleaved with KI-I_2. Judging by the slurred melting point of 110–125°C (Ref. 13, 120°C), the iodoform (methyl carbon of acetate-^{14}C) was impure even after several recrystallizations. The phenylbenzoic acid (carboxyl carbon of acetate-^{14}C) was recrystallized and it melted at 223.5–224°C (unc; Ref. 14, 224°C).

Glycerol-^{14}C from triglyceride-glycerol and from phospholipid-α-glycerophosphate was degraded. The α-glycerophosphate was first purified by column chromatography (15) and then treated with acid phosphatase. The glycerol-^{14}C from each fraction was diluted with carrier glycerol and passed through a mixed bed resin (Bio-Rad AG 501-X8 [D]) to remove ionic contaminants. Periodate degradation (16) of the glycerol-^{14}C yielded carbons 1 and 3 as formaldehyde and carbon 2 as formic acid. The formaldehyde was precipitated as the dimedone derivative (17) (mp 189.5–190°C, unc; Ref. 18, 189–190°C). Formic acid was collected, reduced to formaldehyde (19), and converted to the dimedone derivative. Degradation of commercial glycerol-1,3-^{14}C and glycerol-2-^{14}C after passage through mixed bed resin showed that <0.3% of carbons 1 + 3 contaminated the formic acid from carbon 2 that was reduced to formaldehyde, and <0.1% of carbon 2 contaminated the formaldehyde from carbons 1 + 3.

Fatty acids were separated by gas–liquid chromatography (HI-EFF 2BP on Chromosorb W [AW], Applied Science Laboratories Inc., State College, Pa.). Certain fatty acids were decarboxylated (20) to determine if synthesis was de novo or by elongation.

RESULTS

Incorporation and Distribution of Radiosubstrates

The incorporation of ^{14}C into the aortic lipids is listed in Table 1. The amount of radioactivity incorporated into the lipids of control aortas was often low; extensive

TABLE 1 INCORPORATION OF ^{14}C INTO LIPIDS OF NORMAL AND ATHEROSCLEROTIC RABBIT AORTAS

	Glucose-2-^{14}C	Acetate-1-^{14}C
	*pmoles substrate incorporated/mg wet wt/3 hr**	
Normal	8.6 ± 1.2 (6)	0.22 ± 0.10 (5)
	$P = 0.04$	$P = 0.02$
Atherosclerotic	23.5 ± 6.0 (6)	15.6 ± 5.3 (6)

* Values are means ± SEM, with the number of animals given in parentheses; P values are for differences between normal and atherosclerotic aorta incubated with the same labeled substrate.

analyses were not possible. The production of atherosclerosis caused a significant increase in the amount of both substrates incorporated into lipids.

The distribution of radioactivity among the various lipid classes is shown in Table 2. In each type of aorta, about two-thirds of the label from glucose-2-^{14}C appeared in phospholipids. Atherosclerosis induced no significant changes in the distribution of ^{14}C from glucose-2-^{14}C. Vost (6) found approximately 60% of ^{14}C from glucose-^{14}C in phospholipids and 10–19% in the triglycerides of normal rabbit abdominal aorta after 2 hr of perfusion.

In control aortas, acetate-1-^{14}C was incorporated mostly into phospholipids; the induction of atherosclerosis increased incorporation into triglycerides and cholesteryl esters ($P < 0.001$ for each). The cholesteryl esters were rechromatographed in heptane–ethyl ether 19:1, and the radioactivity moved again to the cholesteryl ester spot; none cochromatographed with squalene. Rao and Rao (21) reported that 14% of acetate-^{14}C was incorporated into normal rabbit aortic triglycerides and 45% into phospholipids; Day and Wilkinson (22) found 10% in triglycerides, 56% in phospholipids, and 29% in cholesteryl esters of atherosclerotic rabbit aortic lipids.

Table 3 lists the amounts of radioactive substrate acetate and glucose incorporated into phospholipids and triglycerides of normal and atherosclerotic aortas. The ratio Atherosclerotic/Normal indicates the increase caused by atherosclerosis. Even though the incorporation of glucose-2-^{14}C into lipids was about the same in normal and atherosclerotic aortas, there was about a threefold increase in substrate incorporation on a per milligram basis into both triglycerides and phospholipids. The increase of acetate-1-^{14}C into both triglycerides and phospholipids was greater than the increase of glucose-2-^{14}C caused by atherosclerosis, but even the maximum incorporation of acetate-1-^{14}C into each of these lipids was not much greater than the amounts of glucose-2-^{14}C incorporated into control aortas.

Distribution of Radioactivity in Lipids

The two radioactive substrates differed markedly with regard to their incorporation into locations within the lipids (Table 4). ^{14}C from glucose-2-^{14}C was present in triglycerides and phospholipids almost exclusively in the glycerol moiety; the incorporation of the radioactive glucose into fatty acids was significantly different ($P = 0.004$) between phospholipids and triglycerides. Acetate-1-^{14}C was incorporated predominantly into fatty acids, with about one-third present in the glycerol moiety of triglycerides and one-fourth in the glycerophosphate of phospholipids. The radioactivity in

TABLE 2 DISTRIBUTION OF SUBSTRATE RADIOACTIVITY INTO LIPIDS OF NORMAL AND ATHEROSCLEROTIC RABBIT AORTA

Lipid Class	Glucose-2-^{14}C		Acetate-1-^{14}C	
	Normal	Atherosclerotic	Normal	Atherosclerotic
		*%**		
Cholesteryl ester	0	1.7 ± 0.3	5.2 ± 1.6	26.9 ± 3.1
Triglyceride	22.0 ± 2.6	26.4 ± 1.3	4.3 ± 1.8	18.3 ± 2.1
Fatty acid	0.3 ± 0.1	0.5 ± 0.1	7.1 ± 1.3	5.8 ± 0.9
Cholesterol + diglyceride	10.4 ± 0.5	5.1 ± 0.2	24.9 ± 8.1	4.1 ± 0.7
Monoglyceride	3.2 ± 0.6	1.7 ± 0.3	9.9 ± 1.9	3.7 ± 0.3
Phospholipid	64.0 ± 2.2	64.6 ± 1.5	48.7 ± 8.0	41.2 ± 1.6

* Percentage of total radioactivity removed from the TLC plates.

TABLE 3 INCORPORATION OF ^{14}C INTO PHOSPHOLIPIDS AND TRIGLYCERIDES OF NORMAL AND ATHEROSCLEROTIC AORTAS

Lipid Class	Substrate Incorporated					
	Glucose-2-^{14}C			Acetate-1-^{14}C		
	Normal	Atherosclerotic	Atherosclerotic/ Normal	Normal	Atherosclerotic	Atherosclerotic/ Normal
	pmoles/mg wet wt/3 hr					
Triglyceride	1.8	6.2	3.4	0.094	2.9	31
Phospholipid	5.48	15.2	2.8	0.107	6.4	59

* Values are products of the percentage incorporation for each lipid class (Table 2) and the total incorporation into lipid (Table 1).

TABLE 4 PERCENTAGE DISTRIBUTION OF RADIOACTIVITY IN LIPIDS OF ATHEROSCLEROTIC RABBIT AORTA

Lipid Class	Glucose-2-^{14}C			Acetate-1-^{14}C		
	Glycerol	Fatty Acid	Non-saponified	Glycerol	Fatty Acid	Non-saponified
	% *					
Triglyceride	96.5	3.3	0.2	31.6	68.3	0.1
Phospholipid	94.0	6.0	0.0	26.1	73.2	0.7
Cholesteryl ester	1.2	98.0	0.8	2.1	97.4	0.5

* Percentage of the sum of the ^{14}C in the three hydrolysis fractions.

cholesteryl esters was present predominantly in fatty acids. Rechromatography of the nonsaponifiable fraction showed slight radioactivity cochromatographing with cholesterol, but this was not characterized further. Vost (6) found 3.3% of the glucose-^{14}C radioactivity in the fatty acids of total lipid, while Day and Wilkinson (22) reported that over 90% of acetate-^{14}C appeared in the phospholipid fatty acids of rabbit aorta.

Presumably, the ^{14}C from either acetate or glucose is present in the aqueous fraction as glycerol or glycerophosphate. Contamination of phospholipid by substrate acetate-1-^{14}C was ruled out by acidifying the water hydrolysate and evaporating it to dryness; over 90% of the radioactivity remained after this treatment, contrary to the loss of added acetate-1-^{14}C in control experiments.

Cholesterol cochromatographs with 1,3-diglycerides on TLC, and since this compound was sometimes incompletely separated from 1,2-diglycerides in aortic lipids from cholesterol-fed animals, these three lipids were considered together (Table 2). With glucose-2-^{14}C as substrate, over 90% of the radioactivity was in the glycerol fraction, indicating predominantly diglycerides. Over half of the ^{14}C from acetate-1-^{14}C was in the fatty acids contained in that spot. After hydrolysis and rechromatography, the amount of radioactivity that cochromatographed with cholesterol would have accounted for <0.6% of the total acetate-1-^{14}C incorporated into aortic lipids.

Table 5 is designed to place in perspective the relative amounts of the two substrates incorporated into the glycerol and fatty acid moieties of phospholipids, triglycerides, and cholesteryl esters by atherosclerotic aortas. The ^{14}C of glucose-2-^{14}C would yield the triose dihydroxyacetone-2-^{14}C phosphate for conversion to glycerol-2-^{14}C. A portion of the triose phosphate isomerizes to glyceraldehyde-2-^{14}C-3-phosphate and provides precursor for acetyl-1-^{14}C CoA used in fatty acid synthesis. Since the specific activity of this acetyl-1-^{14}C CoA is diluted by half of the original glucose-2-^{14}C molecule, the values for fatty acids synthesized from this substrate are doubled in Table 5. No corrections are made for additional dilution by endogenous metabolites, nor are glycerol-^{14}C incorporation values altered because of possible dilution by nonradioactive glyceraldehyde-3-phosphate arising from glucose-2-^{14}C. The activity for glycerol-^{14}C arising from substrate acetate-1-^{14}C is also doubled because of the randomization and loss of ^{14}C that would occur during the conversion of acetate-^{14}C to glycerol-^{14}C. The amount of ^{14}C from glucose-2-^{14}C in the glycerol moiety was 8–15 times more than in fatty acids, whereas acetate-1-^{14}C was distributed almost equally between the glycerol and fatty acid moieties.

Glycerol-^{14}C from the triglycerides and phospholipids was degraded as outlined in the Materials and Methods section. When acetate-1-^{14}C was the substrate, an average of 98.5% of the ^{14}C was in carbons 1 + 3, as would be expected. With glucose-2-^{14}C, 95% of the ^{14}C was in carbon 2 and 5% was randomized into carbons 1 + 3; this is in contrast to 25% or more randomization found in adipose tissue (23) and probably indicates a lower activity of the hexosemonophosphate shunt in aortic tissue than in adipose tissue. Acetate-1-^{14}C arising from glucose-2-^{14}C had >95% of the radioactivity in carbon 1; this establishes the validity of using glucose-2-^{14}C for fatty acid and lipid synthesis.

TABLE 5 COMPARISON OF AMOUNTS OF RADIOACTIVE SUBSTRATE INCORPORATED INTO DIFFERENT MOIETIES OF LIPIDS OF ATHEROSCLEROTIC RABBIT AORTAS

	Total Substrate Incorporation* (Table 1)	Lipid Class	Percentage of Total Lipid† (Table 2)	Total (Table 3)	Glycerol‡	Fatty Acids‡
					pmoles of substrate incorporated/mg wet wt/3 hr	
Glucose-2-^{14}C	23.5	TG	26.4	6.2	6.0	0.2 × 2 = 0.4
		PL	64.6	15.2	14.3	0.9 × 2 = 1.8
Acetate-1-^{14}C	15.6	TG	18.3	2.9	0.90 × 2 = 1.8	2.0
		PL	41.2	6.4	1.7 × 2 = 3.4	4.7

* pmoles of substrate incorporated/mg wet wt of aorta/3 hr.
† Percentage of substrate incorporated into all lipids.
‡ Values are the products of the percentage of each fraction, glycerol or fatty acid, from Table 4 and the absolute amount from Table 3.

TABLE 6 DECARBOXYLATION RATIO* FOR PHOSPHOLIPID FATTY ACIDS OF ATHEROSCLEROTIC RABBIT AORTA

Fatty Acid†	Glucose-2-^{14}C	Acetate-1-^{14}C
14:0	7.0	5.3
16:0	7.9	6.2
18:0	1.1	1.2
18:1	1.2	1.2

* The decarboxylation ratio is total fatty acid radioactivity/carboxyl radioactivity. A ratio of 1.0 indicates that elongation was the only reaction, while a higher ratio indicates increased de novo synthesis, e.g., 7.0 for 14:0 and 8.0 for 16:0.

† Fatty acid = number of carbon atoms:number of double bonds.

Table 6 presents decarboxylation ratios for some of the phospholipid fatty acids synthesized in atherosclerotic aortas. The fatty acids with 14 and 16 carbons arise mainly by de novo synthesis, whereas those with 18 carbons have radioactivity incorporated by elongation, similar to the results of St. Clair, Lofland, and Clarkson (24). Both glucose-2-^{14}C and acetate-1-^{14}C gave similar results.

DISCUSSION

These studies demonstrate the feasibility of using glucose-2-^{14}C to study lipogenesis in normal and atherosclerotic aorta. This substrate is metabolized in a manner consistent with known biochemical pathways. There is little randomization of label; ^{14}C appears predominantly where predicted in glycerol-2-^{14}C and acetate-1-^{14}C. Substrate acetate-1-^{14}C used concomitantly showed that fatty acid synthesis was similar from both labeled substrates. Any estimates on substrate incorporation are minimum values, since the degree of dilution of labeled substrates by endogenous metabolites is unknown. Considering that the radioactive glucose passes through more enzymic steps with more possibilities of endogenous, nonradioactive substrate dilution than the radioactive acetate, the amount of ^{14}C incorporated from glucose-2-^{14}C into fatty acids is quite substantial. This may be physiologically more important due to the greater availability of glucose for in vivo metabolism. In general, there was good agreement between the data obtained here and comparable data from other work (6, 21, 22, 24) in which whole aortic tissue was incubated under similar conditions.

Approximations based on the rates of glyceride-glycerol synthesis from glucose-2-^{14}C and on the amounts of lipid that accrue during the development of atherosclerosis in rabbits fed cholesterol (25–27) indicate that glucose could furnish virtually all of the glycerol in triglycerides and glycerophospholipids synthesized de novo in the aorta. In normal and atherosclerotic rabbit aorta, but not in human aorta (28, 29), the concentration of triglycerides is greater than that of phospholipids; however, the rates of increase due to atherosclerosis are similar. The aortic preparation used here excluded periaortic fat tissues that could have caused contamination with triglyceride-synthesizing enzymes. Numerous workers (5, 6, 22, 30, 31) have noted this problem and have likewise taken care to prevent contamination. The active triglyceride synthesis observed here and elsewhere probably represents metabolism of the aortic intima plus media. Acetate-1-^{14}C incorporation into glyceride-glycerol results from the equilibration of that substrate with endogenous metabolites, but no net glycerol synthesis results. Fatty acids synthesized from either substrate could result in net accumulation.

The incorporation of glucose-^{14}C into cholesteryl esters has not been reported to this extent before, although the substantial incorporation of acetate-1-^{14}C into cholesteryl esters, primarily in the fatty acid moiety, has been reported in this work and that of others (22, 24, 32). The rate of incorporation of acetate-1-^{14}C into the fatty acids that are esterified to cholesterol (Tables 1 and 2) could account for 20% (4) to > 90% (25) of the cholesteryl ester fatty acid that accumulates during the development of atherosclerosis. These data do not differentiate between the net accumulation of cholesteryl esters by the esterification of nascent fatty acids with endogenous cholesterol and the turnover of fatty acids from cholesteryl esters already present in the aorta (32); they merely indicate a potential for net accumulation.

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