Acetate-1-^{14}C incorporation into polyunsaturated fatty acids of phospholipids of developing chick brain

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ABSTRACT The incorporation of acetate-1-^{14}C into the polyunsaturated fatty acids of glycerophosphatides of chick embryonic brain has been studied. After the injection of acetate-1-^{14}C into the yolk sac, differences were found in the degree of labeling of the major fatty acids of the ω3 and ω6 series. Arachidonic acid (20:4ω6) showed a high degree of radioactivity while docosahexaenoic acid (22:6ω3) was poorly labeled, at a period of brain development when both fatty acids were being actively deposited. Evidence is presented to indicate that the low activity in docosahexaenoic acid is not explicable on the basis of either a low or high rate of turnover of this polyenoic acid. Similar results were obtained whether the rapid early or slower late stage of brain development was examined. It is suggested that the elongation of ω3 and ω6 series acids may be under the control of different regulatory mechanisms.

KEY WORDS polyunsaturated fatty acids · ω3 and ω6 · biosynthesis · acetate · developing chick brain · arachidonic acid · docosahexaenoic acid · glycerophosphatides

THE POLYUNSATURATED fatty acids (PUFA) of the ω6 and ω3 series[1] can be derived from exogenous 18:2 and 18:3, respectively, in the developing chick brain (1), as they are in mammalian tissue (2, 3). The yolk, which is the sole nutritive source for the chick embryo, contains only trace amounts of 18:3, while 22:6ω3 is one of the two major PUFA of brain glycerophosphatides in chick as well as mammalian brain (4, 5). Since apparently insufficient 18:3 is available for elongation to 22:6, the biosynthesis and turnover of this latter PUFA in chick developing brain have been investigated. It has been shown that other fatty acids, and presumably 22:6, can be oxidized by brain slices (6, 7) and that there is a turnover of brain phospholipids in the adult (8). While some or perhaps all of the requirements of the embryo for 22:6 could be satisfied by transport of this PUFA from the yolk, the mechanism of biosynthesis of this long-chain PUFA in the brain in the postembryonic period after the blood–brain barrier has developed requires elucidation. The present study is concerned with the embryonic development period.

METHODS

Fertile White Leghorn eggs were incubated at constant temperature (37°C) and humidity (68%). At selected periods during development, sodium acetate-1-^{14}C (2.0 mc/mmole, New England Nuclear Corp., Boston, Mass.) was injected into the yolk sac. 10 μc was used at an early stage of growth; at the late developmental period 100 μc was injected. The embryos were sacrificed at appropriate time intervals after the administration of the isotope; the brains were removed, homogenized in 0.9% saline, and lyophilized immediately.

The lipids were extracted with $CHCl_3$–CH_3OH 2:1, and the glycerophosphatides were separated from the neutral lipids by silicic acid column chromatography (9). The phospholipid fractions were methanolyzed with 5% H_2SO_4 in methanol, and the fatty acid esters were

Abbreviation: PUFA, polyunsaturated fatty acids. Fatty acids are designated by number of carbon atoms: number of double bonds.

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[1] ω3 and ω6 refer to the position of the first double bond, counting from the methyl end of the molecule.

separated into three fractions on a silver nitrate–silicic acid column (10). The saturated, monoenoic, and dienoic esters were removed as described previously (4) and the PUFA fraction was eluted from the column with methanol–benzene 3:7. The fatty acid composition of the PUFA was determined on a dual-column Packard gas chromatography equipped with a radium detector, with temperatures programmed from 60° to 190°C at 4°/min. Gas-Pack P (Applied Science Laboratories Inc., State College, Pa.) was used as the solid support with 12% diethylene glycol succinate polyester as the liquid phase. Each fatty acid was collected on a cartridge containing recrystallized *p*-terphenyl coated with 5% Dow Corning 550 silicone fluid as described by Karmen, McCaffrey, Winkleman, and Bowman (11). Radioactivity was determined in a Packard Tri-Carb scintillation spectrometer with 0.5% diphenyloxazole:0.03% 1,4-bis[2-(5-phenyloxazolyl)]benzene in toluene as the scintillation mixture.

Oxidative ozonolysis was performed as follows: 1–3 mg of unlabeled PUFA methyl esters was added to the labeled PUFA fraction, which was saponified and acidified. The fatty acids were extracted with ethyl acetate and ozonized with the aid of an ozone generator as described by Kishimoto and Radin (10), and the ozonides were degraded by means of 0.1 ml of 98% HCOOH and 0.05 ml of H_2O_2 per 2 ml of ethyl acetate, with the use of a solid CO_2–acetone bath for cooling and KI-starch as an indicator. The degradation products were methylated with 14% BF_3 in CH_3OH (Applied Science Laboratories, Inc.) at 70°C for 10 min.

Linolenate-1-^{14}C was obtained from Nuclear-Chicago Corporation, Des Plaines, Ill.; as determined by dilution analysis of the 9,10,12,13,15,16-hexabromostearic acid it had a radiopurity of 89%, and a chemical purity of 99% by acid value determination.

The mono- and dicarboxylic acids derived from the ozonides were identified by comparison of their retention times with those of known compounds except for undecanoic acid, which was identified from a semilogarithmic plot of retention time vs. carbon number.

RESULTS

In the early period of development (Table 1, Experiment 1), 5 days after acetate-1-^{14}C administration to the 0-day embryo, most of the radioactivity in the PUFA fraction was found in 20:4ω6 and 22:4ω6, which contained 30.5 and 32.7% of the total PUFA label, respectively. In contrast, the ω3 acids (20:5, 22:5, and 22:6) contained only 2.6–4.7% of the total PUFA label. Since 20:5 and 22:5 are present in trace amounts, it can be presumed that despite the low absolute activity, the specific activity may be high. However, although 22:6ω3 is a major component and is actively deposited in the brain during this developmental period, only a low rate of incorporation of acetate-1-^{14}C into this molecule is evident.

To ascertain whether the low activity of 22:6ω3 could result from a rapid turnover of this fatty acid, in which after 5 days the initially labeled molecule had been replaced by unlabeled material, we performed a short-term incubation experiment. At the earliest possible time at which it was technically feasible to collect material, namely 5–6 days, brains were obtained 13 hr after the injection of acetate-1-^{14}C. The amount of

TABLE 1 Fatty Acid Composition* and Acetate-1-^{14}C Incorporation† into Polyunsaturated Fatty Acids (PUFA) of Glycerophosphatides of Chick Embryonic Brain

Day Injected	Incubation Time after Injection	Incorp. into PUFA‡	PUFA													
			20:3§		20:4ω6		20:5ω6§		22:4ω6		22:5ω6		22:5ω3		22:6ω3	
			Amt.	Act.	Amt.	Act.	Amt.	Act.	Amt.	Act.	Amt.	Act.	Amt.	Act.	Amt.	Act.
			% of total PUFA fraction													
Expt. 1																
0	5 days	1950	1.7	10.0	49.4	30.5	tr.	4.0	10.3	32.7	9.7	4.3	tr.	4.7	26.1	2.6
6	13 hr	350	2.3	10.9	52.2	25.2	tr.	7.2	8.2	32.4	6.7	8.4	tr.	4.3	29.2	3.1
Expt. 2																
10	28 hr	3950	1.8	5.4	46.0	29.4	tr.	4.4	10.7	28.1	6.8	11.9	tr.	5.2	33.0	4.4
10	5 days	1690	1.0	4.2	44.3	28.7	tr.	2.7	5.5	26.8	6.0	16.5	tr.	4.8	44.3	8.1
16	26 hr	1040	1.2	8.3	44.2	30.0	tr.	4.2	6.2	22.1	9.2	13.1	tr.	3.4	39.4	6.7

Expt. 1: 10 μc of acetate-1-^{14}C was injected into the yolk sac (early development stage). Expt. 2: 100 μc was injected (late stage of development). At appropriate periods, embryos were sacrificed and the glycerophosphatides of brain were separated on a silicic acid column. Methyl esters of fatty acids of glycerophosphatides were separated on an $AgNO_3$–silicic acid column. Saturated acids were eluted with benzene–petroleum ether 6:94; mono- and dienoic acids with benzene–petroleum ether 1:1; and polyenoic acids mixture (PUFA) with methanol–benzene 3:7. The PUFA fraction was analyzed by GLC.

* Area percentage of total PUFA on GLC analysis.

† Radioactivity percentage recovered in PUFA fraction by GLC collection.

‡ Activity is expressed as cpm/μatom of glycerophosphatide-P.

§ Identification of these compounds is tentative.

TABLE 2 DISTRIBUTION OF RADIOACTIVITY IN OZONIDE DEGRADATION PRODUCTS DERIVED FROM GLYCEROPHOSPHATIDE POLYENOIC ACIDS OF CHICK EMBRYONIC BRAIN AFTER INCUBATION WITH ACETATE-1-^{14}C

Product	Radioactivity
	%
Monocarboxylic acids	
C_6	1.9
C_7	1.1
C_8	2.0
Dicarboxylic acids	
C_3	—
C_4	2.3
C_5	16.6
C_6	7.3
C_7	37.8
C_8	4.7
C_9	8.1
C_{10}	3.1
C_{11}	5.5

acetate found to be incorporated into 22:6ω3 did not differ appreciably from that observed in the earlier longer-term experiment.

The relative amounts of incorporation of acetate-1-^{14}C into PUFA at a later stage of development showed (Table 1, experiment 2) only one significant change from the results obtained at the early stage: an increased incorporation into 22:5ω6. The brain continued to be unable to incorporate acetate into 22:6ω3 despite an increase in the concentration of this PUFA from 26.1% of the PUFA fraction at 5 days to 39.4% at 16 days of age. During this period the phospholipid phosphorus increased from about 1 μmole/brain to about 15 μmoles/brain (4). Arachidonic acid, the other principal PUFA in brain, showed no appreciable changes either in percentage of PUFA or in the relative amount of acetate incorporated from the early to the later developmental stage.

After oxidative ozonolysis, the major radioactivity was recovered in the C_5- and C_7-dicarboxylic acids, which are presumably derived from the carboxyl end of 20:4ω6 and 22:4ω6, respectively (Table 2). Essentially no activity could be recovered in the C_6-monocarboxylic acid isolated from the methyl end of ω6 series acids. Apparently PUFA of the ω6 series are formed by elongation and not to any appreciable extent by de novo synthesis from acetate fragments; this finding is in agreement with that of Klenk on rat liver (12).

Only trace amounts of 18:3 are present in the yolk, while 11.8% of the glycerophosphatide fatty acids and 16.9% of the free fatty acids of yolk consist of 18:2 (4). Mohrhauer and Holman (13) have shown in feeding experiments that a competitive inhibitory effect exists between 18:2 and 18:3. To ascertain whether this mechanism could be operative in producing a low rate of incorporation into 22:6ω3, we injected acetate-1-^{14}C together with unlabeled 18:3 into 2-day eggs and sacrificed the embryos 5 days later. Results of this experiment are shown in Table 3, together with those obtained when acetate-1-^{14}C and linolenate-1-^{14}C alone were injected [the latter results have been reported previously (14)].

With acetate-1-^{14}C, the results obtained were essentially the same as those in experiment 1 (Table 1). The uptake of 18:3ω3 into the acids of ω3 series is apparent; 21.9% of the PUFA activity appeared in 22:6 compared with 3.0% when acetate-1-^{14}C was used. When acetate-1-^{14}C was injected together with 5 μmoles of unlabeled linolenate, the distribution of PUFA and the uptake of acetate-1-^{14}C by the individual fatty acids appeared to be the same as when acetate alone was used. When 20 μmoles of linolenate was used, 20:4 decreased from 51.7 to 30.4% and 22:6 increased from 33.5 to 45.8%. The levels of some of the minor PUFA also changed, but not as markedly as those of the two

TABLE 3 EFFECT OF LINOLENATE ON FATTY ACID COMPOSITION* AND ON ACETATE-1-^{14}C INCORPORATION† INTO PUFA OF GLYCEROPHOSPHATIDES OF CHICK EMBRYONIC BRAIN

	Incorp. into PUFA‡	18:3 Area		20:3§		20:4ω6		20:5ω3§		22:4ω6		22:5ω6		22:5ω3		22:6ω3	
		Amt.	Act.	Amt.	Act.	Amt.	Act.	Amt.	Act.	Amt.	Act.	Amt.	Act.	Amt.	Act.	Amt.	Act
									% of total PUFA fraction								
Acetate-1-^{14}C	1287	tr.	5.4	1.9	8.2	51.7	37.9	tr.	5.7	7.4	27.7	5.4	7.6	tr.	4.5	33.5	3.0
Linolenate-1-^{14}C	920		5.5		0.9		10.7		15.2		4.9		6.0		35.0		21.9
Acetate-1-^{14}C + linolenate 5 μmoles	1289	tr.	3.8	4.4	6.5	50.0	37.4	tr.	3.9	8.5	32.0	2.6	7.3	tr.	4.3	33.0	3.4
+ linolenate 20 μmoles	1488	tr.	4.6	1.6	7.7	30.4	37.0	tr.	4.0	11.7	28.9	5.1	6.3	1.6	4.1	45.8	5.0

Acetate-1-^{14}C, linolenate-1-^{14}C (10 μc/egg), and unlabeled linoleanate were injected (with egg-white) into the yolk sac of the 2-day eggs. In the control experiments egg-white alone was injected. Embryos were sacrificed 5 days later and the separation procedures were the same as in Table 1. The data for linolenate-1-^{14}C are from an earlier experiment (14).

*, †, ‡, § Footnotes as in Table 1.

major PUFA. Despite this effect on the concentration of the individual PUFA, the amount of acetate incorporated into 20:4 and 22:6 did not change appreciably from that observed with acetate-1-^{14}C alone.

DISCUSSION

It has been demonstrated that linolenate-1-^{14}C can serve as a precursor for 22:6 in chick embryonic brain. When the amounts of 18:3 available are inadequate, it is conceivable that the requirements for 22:6 in the developing chick embryo can be supplied by preformed 22:6 from the yolk (15). Nevertheless, when 18:3 is supplied exogenously, it is converted to 22:6. This conversion does not appear to involve a concomitant labeling of the hexaenoic acid with acetate-1-^{14}C to any appreciable extent. Klenk (16) derived a C_4-dicarboxylic acid containing radioactive acetate from the PUFA fraction of rat liver slices but this fragment could have originated from the 22:5ω6 acid since the 22 acids were treated in toto. Mohrhauer and Holman (17) have shown in liver slice experiments that acetate-1-^{14}C is incorporated into 22:5ω3 but not into 22:6ω3, results that are similar to those reported here. They have suggested that these findings indicate a low rate of turnover for this terminal metabolite in the ω3 series of PUFA. However, when linolenate-1-^{14}C is used as a precursor, an active uptake into 22:6ω3 can be seen, which seems to indicate at least the linolenic acid label is utilized. Thus the amount of linolenate-1-^{14}C incorporated into 22:6 is higher than that of acetate-1-^{14}C, despite the fact that elongation by acetate requires two units for each linolenate molecule.

It can be noted that despite its low concentration, 22:5ω3 showed a relatively high specific activity with acetate-1-^{14}C, and incorporated more linolenate-1-^{14}C than 22:6. If the system of elongation from 18:3 to 22:5 proceeds via acetate as has been postulated (18), the synthesis of 22:6 from 22:5 may be regulated by a low activity of the desaturase (or cofactors) in this equilibrium; alternatively, the disproportionate levels of these two metabolites may tend to shift the equilibrium to the left and thus limit 22:6 production.

The chick embryo plus yolk constitutes a closed system in which labeled acetate can be recycled and is available for an extended time period. It would be expected, since there is a net synthesis of 22:6, that in the longer incubation periods, 22:6 would have a higher activity than that observed during a short incubation period. This is borne out by Table 1, experiment 2, in which the percentage incorporation into PUFA is increased from 4.4% after 28 hr of incubation to 8.1% at 5 days. It is difficult to determine the significance of this increase. It could be expected that the specific activity would approach that of 22:5 under these conditions, but this is not so. Whether 22:5 is being utilized in other pathways or whether it is an intermediate in 22:6 biosynthesis in chick brain is undetermined. Korn (19) has postulated the 22:5ω3 to 22:6ω3 conversion in protists.

Dilution of acetate-1-^{14}C by a large unlabeled pool could result in a low level of radioactivity in 22:6. If the dilution effect were pronounced, one would expect 20:4 to be similarly affected by such dilution, since at a late developmental stage 20:4 and 22:6 are being deposited at approximately the same rate in the PUFA fraction. Different rates of turnover may account for some of the differences observed for these two acids. The data obtained with linolenate are not consistent with a low turnover for 22:6. Furthermore, relatively large amounts of 22:6 are deposited at this rapidly growing stage of the embryonic brain. Thus a low turnover of 22:6 is probably excluded as an explanation for the low incorporation of acetate; a rapid turnover is ruled out by the short-term experiments, for they yielded the same results as the longer-term experiments.

That linolenate can be incorporated into 22:6 at a time when very little acetate is being incorporated into this long-chain hexaenoic acid is thus difficult to explain. A low rate of turnover could not account for these results unless different portions of the fatty acid have different turnover rates, and the low acetate incorporation seems to be due neither to a low nor to a high rate of turnover. It would appear that the elongation mechanisms for the ω3 and ω6 series from linolenic and linoleic acid, respectively, may differ and may involve specific regulatory factors. Separate compartmentalization of these two fatty acids could explain the differences in elongation reported here. In any event, the mechanism of the elongation of 18:3ω3 to 22:6ω3, as well as the nature of the precursor of the latter in brain, requires further study.

The utilization of acetate for elongation is unaffected by the competitive mechanism between 18:2 and 18:3, since the addition of 18:3 did not affect acetate-1-^{14}C incorporation into 20:4. This tends to support our suggestion that acetate is not as readily available for elongation in the ω3 series as in the ω6 series. Also, after incubation with linolenate-1-^{14}C, 10.7% of the label was recovered in arachidonic acid. This could be attributed possibly to the degradation of linolenate by β-oxidation during the 5 day incubation period and the subsequent incorporation of the small fragments into ω6 biosynthetic pathways.

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