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Arachidonic and docosahexaenoic acids are biosynthesized from their 18-carbon precursors in human infants

(polyunsaturated fatty acids/elongation/desaturation/fatty acid metabolism/infant nutrition/gas chromatography/mass spectrometry)

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ABSTRACT **It is becoming clear that an adequate level of long-chain highly unsaturated fatty acids in the nervous system is required for optimal function and development; however, the ability of infants to biosynthesize long-chain fatty acids is unknown. This study explores the capacity of human infants to convert 18-carbon essential fatty acids to their elongated and desaturated forms, *in vivo*. A newly developed gas chromatography/negative chemical ionization/mass spectrometry method employing ^{2}H-labeled essential fatty acids allowed assessment of this *in vivo* conversion with very high sensitivity and selectivity. Our results demonstrate that human infants have the capacity to convert dietary essential fatty acids administered enterally as ^{2}H-labeled ethyl esters to their longer-chain derivatives, transport them to plasma, and incorporate them into membrane lipids. The *in vivo* conversion of linoleic acid (18:2n6) to arachidonic acid (20:4n6) is demonstrated in human beings. All elongases/desaturases necessary for the conversion of linolenic acid (18:3n3) to docosahexaenoic acid (22:6n3) are also active in the first week after birth. Although the absolute amounts of n-3 fatty acid metabolites accumulated in plasma are greater than those of the n-6 family, estimates of the endogenous pools of 18:2n6 and 18:3n3 indicate that n-6 fatty acid conversion rates are greater than those of the n-3 family. While these data clearly demonstrate the capability of infants to biosynthesize 22:6n3, a lipid that is required for optimal neural development, the amounts produced *in vivo* from 18:3n3 may be inadequate to support the 22:6n3 level observed in breast-fed infants.**

Polyunsaturated acids of the n-6 series are essential for proper growth and development (1). This may be supplied as linoleic acid (18:2n6), although arachidonic acid (20:4n6) may also be required (2). Recent evidence derived from non-human primates suggests that n-3 fatty acids are also essential for optimal neural development (3). In human infants, decreases in the level of plasma and erythrocyte membrane docosahexaenoate (22:6n3) are associated with poorer retinal development (4) and lower visual acuity as measured behaviorally by forced choice preferential looking testing and electrophysiologically using pattern reversal visual evoked potential methodology (5). Cognitive scores at 12 months are also lower in infants fed docosahexaenoate-deficient formula (6). This neural n-3 deficiency syndrome has been the subject of several recent reviews (7–10). Carlson *et al.* (11) have suggested that adequate 20:4n6 is also necessary for optimal growth and cognition. Birch *et al.* (5) found that a diet high in the n-3 precursor linolenate (18:3n3) was not able to support optimal visual function and that the supply of preformed 22:6n3 appeared necessary in premature infants (5).

These studies underline the importance of understanding the metabolism of the 18-carbon essential fatty acids to their elongated and desaturated forms *in vivo*, particularly of 20:4n6 and 22:6n3. However, not only is there a complete lack of information concerning essential fatty acid metabolism in early human development but there is also a paucity of information in adults and in other primates. *In vitro* experiments have included the demonstration of Δ6 desaturation of ^{14}C-labeled 18:2n6 or 18:3n3 and the Δ5 desaturation of 20:3n6 in liver microsomes from human infants (12) as well as Δ6 and Δ5 desaturation of 18:3n3 and 20:3n6 in human lymphocytes (13). Several *in vivo* experiments concerning fatty acid elongation/desaturation have been attempted. In 1967, Nichaman *et al.* (14) gave an oral dose of ^{14}C-labeled 18:2n6 to four adult males and found the plasma radioactivity mainly in the form of esterified 18:2n6 but reported traces of radioactive 20:3n6 and 20:4n6 in plasma phospholipids. Emken and coworkers (15, 16) have orally administered stable isotope-labeled 18:2n6 and 18:3n3 and clearly demonstrated formation of deuterated plasma 22:6n3 *in vivo* in adult humans. However, no conversion of 18:2n6 to 20:4n6 was observed in these two studies. El Boustani *et al.* (17) have observed the conversion of deuterated 20:3n6 to 20:4n6 *in vivo* in four adults.

In this study, the ability of human infants to elongate and desaturate essential fatty acids *in vivo* has been examined using a highly sensitive and selective gas chromatography/mass spectrometry method (18) for the detection of stable isotope-labeled metabolites released into plasma subsequent to an oral dose of deuterated fatty acids. Evidence for the enzymatic capacity for the *in vivo* production of 20:4n6 and 22:6n3 in newborn infants is presented.

MATERIALS AND METHODS

Materials. Deuterated linoleic ethyl ester ($^2H_5$-17,17,18,18,18-18:2n6) and deuterated linolenic ethyl ester ($^2H_5$-17,17,18,18,18-18:3n3) from Cambridge Isotope Laboratories (Woburn, MA) were analyzed by GC/flame ionization detector, by GC/MS, and by elemental composition and were found to be of >98% chemical purity.

Clinical. This project was approved by the Institutional Review Board of the National Institute of Alcohol Abuse and Alcoholism, National Institutes of Health, and the Ethics Committee at Instituto de Nutrición y Tecnología de los Alimentos (INTA) that regulate the use of humans as experimental subjects. In addition, this study was performed in conformity with the requirements of the Office for the Protection of Research Risks, Department of Health and Human Services, for international collaborations by means of a Single

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Abbreviations: GC/NCI/MS, gas chromatography/negative chemical ionization/mass spectrometry.

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Project Assurance. Written informed consent was obtained from the infant's parents in Santiago, Chile. Also, the administration of deuterated fatty acids was approved by the Center for Food Safety and Applied Nutrition, Food and Drug Administration.

Infants in this study remained hospitalized after birth due to mild to moderately severe respiratory illness and/or were being observed for possible complications resulting from neonatal asphyxia (Table 1). Routine neonatal care and treatment were provided by a neonatologist from the Hospital Sotero del Rio in Santiago, Chile. Entry criteria included not being enterally fed at time of study and anticipated tolerance to enteral feedings, not being expected to require parenteral lipids during the course of the study, good glucose tolerance, absence of metabolic acidosis, and normal liver enzyme levels. Medications used in the customary care of infants recovering from asphyxia such as antibiotics or phenobarbital or an increase in ambient oxygen were not reasons to exclude infants from the study. In addition, the clinical caretaker and the parents agreed to include the infant in the study. Subjects were given 0.5 ml of a neat solution containing 50 or 100 mg of D_5-labeled 18:2n6 and/or D_5-labeled 18:3n3 per kg of birth weight via a nasogastric tube over a 1-min period; 5 ml of sterile water was used to rinse the tube, which was then occluded to prevent loss of the label.

The nine infants admitted to the study had birth weights ranging from 1980 to 3970 g and gestational ages of 32–41 weeks. Feedings were uncontrolled and consisted principally of human milk but also of commercial formulas; feeds were generally started at least 24 hr (range, 8–96 hr; Table 1) after isotope administration based on the decision of the physician providing clinical care. D_5-fatty acid administration was performed on day 1–6 after birth. Then, at various times, ≈0.5 ml of blood was drawn from an antecubital vein into plastic tubes containing EDTA as anticoagulant; a maximal amount of 3.0 ml was drawn in total. The blood was separated immediately into packed red blood cells and plasma by centrifugation at 3000 rpm for 10 min in a clinical centrifuge. The plasma was pipetted into a separate tube and frozen at −80°C until analysis.

Fatty Acid Extraction and Derivatization. Internal standard (0.05 μg of 23:0 methyl ester) was added to 0.20-ml plasma aliquots, and the lipids were extracted and the pentafluorobenzyl esters were analyzed by GC/MS in the negative chemical ionization (NCI) mode using methods previously described (18, 19).

RESULTS

When the ethyl ester forms of $^{2}H_5$-18:2n6 and $^{2}H_5$-18:3n3 were given by gavage to infants, they were rapidly absorbed and detected in plasma lipids. The dosage and type of each ^{2}H-labeled fatty acid given as well as information about the feeding of each infant is given in Table 1. In all cases, the $^{2}H_5$-18:2n6 levels peaked in the 1–4 μg/ml plasma range within the first 24 hr (Fig. 1*A*). The plasma levels of $^{2}H_5$-18:3n3 were more variable and three of the seven infants exhibited a rather low accretion of this n-3 precursor (Fig. 1*B*). The precise time of the maxima for essential fatty acid absorption was not determined, as the primary purpose of this work was to detect longer-chain metabolites that have maximas at later time points; the need to minimize blood draws for subject protection precluded obtaining additional time point data. However, it appeared that the 18:3n3 absorption peak was faster than that of 18:2n6 in these infants.

Since our GC/MS method is capable of separation of the $^{2}H_5$-labeled fatty acids from their endogenous counterparts in time and by mass, the *in vivo* formation of long-chain polyunsaturates from 18-carbon precursors can be readily observed by inspection of the appropriate selected ion chromatograms (Fig. 2). It is clear from Fig. 2*A* that $^{2}H_5$-20:4n6 is present in infant plasma and must have been formed *in vivo* from the $^{2}H_5$-18:2n6 given. Similarly, Fig. 2 *B* and *C* show the separation of $^{2}H_5$-20:5n3 and $^{2}H_5$-22:6n3 from endogenous peaks related to these n-3 fatty acids, respectively, and these could only have been formed from the *in vivo* conversion of the $^{2}H_5$-18:3n3 given orally to the infant. Plots of the "time 0" chromatograms, representing the responses of the respective $^{2}H_5$ channels prior to dosing with the stable isotopes, demonstrated that the signal-to-noise ratio was quite high. This clearly shows that the peaks indicated represent endogenous conversion of 18:2n6 to 20:4n6 and of 18:3n3 to 22:6n3. The quantity of the $^{2}H_5$-20:5n3 was the greatest of all of the isotopically labeled metabolites in all infants studied (Fig. 2*B*). Other fatty acids for which ^{2}H-labeled peaks were measured in most subjects included the following: 20:3n3, 22:5n3, 18:3n6, 20:2n6, 20:3n6, and 22:4n6.

The time course of the rise and decay for $^{2}H_5$-20:4n6, $^{2}H_5$-20:5n3, $^{2}H_5$-22:5n3, and $^{2}H_5$-22:6n3 plasma levels for each individual infant is shown in Fig. 3. In one infant, no deuterated 20:4n6, 22:5n3, or 22:6n3 was observed but a relatively small amount of labeled 20:5n3 was formed. This infant displayed a relatively high level of plasma $^{2}H_5$-18:2n6 but very low levels of $^{2}H_5$-18:3n3; the absence of the $^{2}H_5$-20:4n6 peak thus indicates low metabolic capacity. The maximal levels of the $^{2}H_5$-labeled products observed were 107, 407, and 224 ng/ml of plasma for $^{2}H_5$-20:4n6, $^{2}H_5$-20:5n3, and $^{2}H_5$-22:6n3, respectively. Peak levels of plasma $^{2}H_5$-20:4n6 and $^{2}H_5$-22:6n3 occurred at 96–144 hr, whereas $^{2}H_5$-20:5n3 peaked at about 24 hr.

The concentrations of plasma fatty acids were determined at the beginning and end of the experiment for each infant, during which time enteral feedings were initiated. The mean plasma concentrations of 18:2n6 at the beginning and end of the experiment were 95 and 372 μg/ml; those for 18:3n3 were

Table 1. Clinical and experimental data

Subject*	Birth weight, g	Gestational age,† sex	Dose, mg/kg $^{2}H_5$-18:2n6	Dose, mg/kg $^{2}H_5$-18:3n3	Fed, hr after isotope‡	Diagnosis
s1	2350	38, ♀	100	100	96	Birth asphyxia, meconium aspiration
s2	3970	39, ♂	100	100	24	Transient tachypnea
s3	2090	35, ♂	100	100	24	Birth asphyxia, hyaline membrane
s4	2080	33, ♂	100	100	48	Birth asphyxia, hyaline membrane
s5	3270	39, ♀	100	50	24	Hyperbilirubinemia, pneumonia
s6	1980	32, ♂	100	50	24	Wet lung, hyperbilirubinemia
s7	2240	35, ♂	100	0	51	Hyaline membrane
s8	3500	40, ♀	100	0	8	Birth asphyxia
s9	3000	41, ♂	0	100	24	Birth asphyxia, meconium aspiration

*Subject numbers used are conserved in the figures.

†Gestational age is in weeks.

‡Number of hours after introduction of the stable isotopes in which enteral feedings were begun.

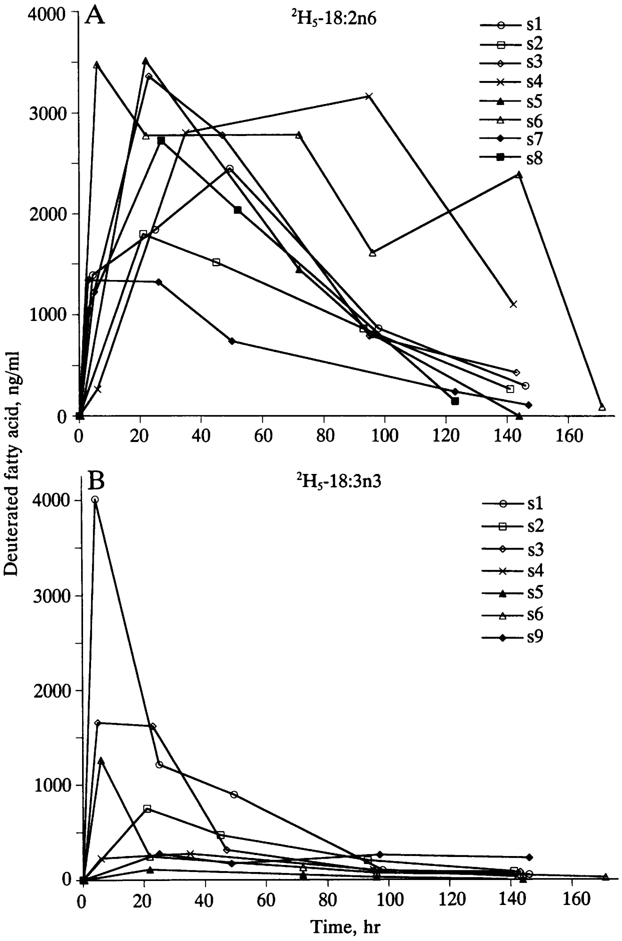

FIG. 1. Time course of deuterated linoleic (18:2n6) and linolenic (18:3n3) acid appearance and decay in infant plasma after a single oral dose. Data represent the absolute quantity of the deuterated fatty acids indicated, in ng/ml of plasma. (*A*) $^2H_5$-18:2n6. (*B*) $^2H_5$-18:3n3. The same symbol is used for each subject in all figures.

0.9 and 3.2 μg/ml. The plasma concentrations of long-chain polyunsaturates were altered little during this period; the mean concentrations at the end of the experiment for 20:4n6, 20:5n3, 22:4n6, 22:5n3, and 22:6n3 were 94, 5.1, 4.7, 4.1, and 42 μg/ml. The initial and final plasma n-6/n-3 ratios were 3.8 and 9.6, respectively.

DISCUSSION

These data unambiguously demonstrate the conversion of 18:2n6 to 20:4n6 and 18:3n3 to 22:6n3 in human infants; this study is also the largest human study yet reported. Many investigators have attempted to study the *in vivo* conversion of 18-carbon essential fatty acids based upon alterations in fatty acid composition when various dietary changes are made. However, compositional studies cannot be interpreted in this manner due to possible exchanges of fatty acids between organs (20). In this study, the precursor fatty acids were isotopically labeled with ^2H atoms and they, as well as their metabolites, were thus distinguishable from the corresponding endogenous molecules when highly specific mass spectrometric detection was used (18). It is thus certain that elongation and desaturation of 18:2n6 and 18:3n3 occurred *in vivo* in these infants.

This study reports direct evidence of 20:4n6 formation from 18:2n6 *in vivo* in humans (Figs. 2*A* and 3*A*). Support for this finding is gained from previous findings that human fetal (21) or adult (22) liver microsomes are capable of 18:2n6 conversion to 20:4n6. Also, 20:3n6 may be converted to 20:4n6 in liver microsomes from human infants (12) and *in vivo* in adults (17). Emken and coworkers (15, 16) have previously demonstrated that 22:6n3 is formed from 18:3n3 *in vivo* in a small number (two to four) of adults; however, they concluded that "The absence of detectable levels of 20:4 indicates that the rate of conversion of 18:2-$^2H_4$ to 20:4-$^2H_4$ is extremely low in normal subjects" (15). This apparent contradiction is attributed to the increased detectability achieved in the present study due to three factors: (*i*) increased sensitivity and selectivity due to complete gas chromatographic separation of the deuterated fatty acid from the endogenous peak due to the presence of five ^2H atoms on the methyl end of the molecule, (*ii*) the increase in sensitivity of several orders of magnitude and increased signal-to-noise ratio gained when using negative ion detection of the pentafluorobenzyl derivatives (18), and (*iii*) blood sampling at time points greater than 48 hr, which, as shown in this study, is necessary in order to detect the maximal level of $^2H_5$-20:4n6 in plasma. The introduction of a period of fasting just prior to the isotope feeding may also be an important factor. In more recent work, Emken *et al.* (23) have presented data for the total n-6 metabolite production from deuterated-18:2n6 but have not shown any direct evidence for 20:4n6 production *in vivo*.

Although the plasma levels of $^2H_5$-20:4n6 were generally $<$100 ng/ml (Fig. 3*A*), it is not correct to interpret this as a very low rate of 18:2n6 conversion. Conversely, although the amounts of total long-chain n-3 metabolites reached 540 ng/ml, it does not follow that the rate of 18:3n3 conversion is much greater than that of the n-6 family. The plasma specific activity is affected by the degree of absorption, elongation/desaturation, oxidation and other degradative processes, transport and removal from the plasma compartment to organs, and transacylation. The isotopically labeled fatty acids will be diluted into the endogenous pools in the liver and plasma and be representative of these pools based on the amount of this dilution. The differences in pool size between the endogenous 18:2n6 and 18:3n3 must therefore be taken into account. The mean plasma concentrations of 18:2n6 and 18:3n3 in these infants were 372 μg/ml and 3.2 μg/ml (at the end of the experiment), respectively, and assuming a similar volume of distribution thus represents a 116-fold difference in estimated pool size. Indeed, when this factor is considered, a more reasonable conclusion is that the rate of n-6 conversion is greater than that of the n-3 family.

An estimation of the total amount of long-chain polyunsaturated fatty acid metabolite formed may be made as follows: (*i*) the total amount of plasma deuterated precursor (i.e., 18:2n6 or 18:3n3) is estimated by integrating its time course plot, (*ii*) the total amount of endogenous plasma precursor fatty acid is estimated from integrating a two-point plot of its time course, (*iii*) the isotope dilution factor in the plasma may be computed from the ratio of the above, (*iv*) the total amount of deuterated long-chain polyunsaturated fatty acid plasma metabolites may be estimated from the integration of their time course plots, (*v*) multiplication of the precursor isotope dilution factor by the total amount of deuterated metabolite then yields a minimal value for the total amount of metabolite produced in the time period of the experiment. If this method of estimation is used for subject 3 (s3), the quantities produced over the 6-day period of the experiment for 20:3n6, 20:4n6, 20:5n3, 22:5n3, and 22:6n3 are approximately 110, 53, 1.3, 0.3, and 0.9 mg. This example illustrates the powerful effect on the conclusions drawn when the differences in the endogenous pools of 18:2n6 and 18:3n3 are considered. The limitations of this estimate must also be explicitly recognized. It assumes that the isotope is homogeneously diluted into the plasma pool and that this dilution is maintained in the liver where enzymatic conversion occurs. The amounts calculated are minimums as only the plasma pool is measured and organ pools are not

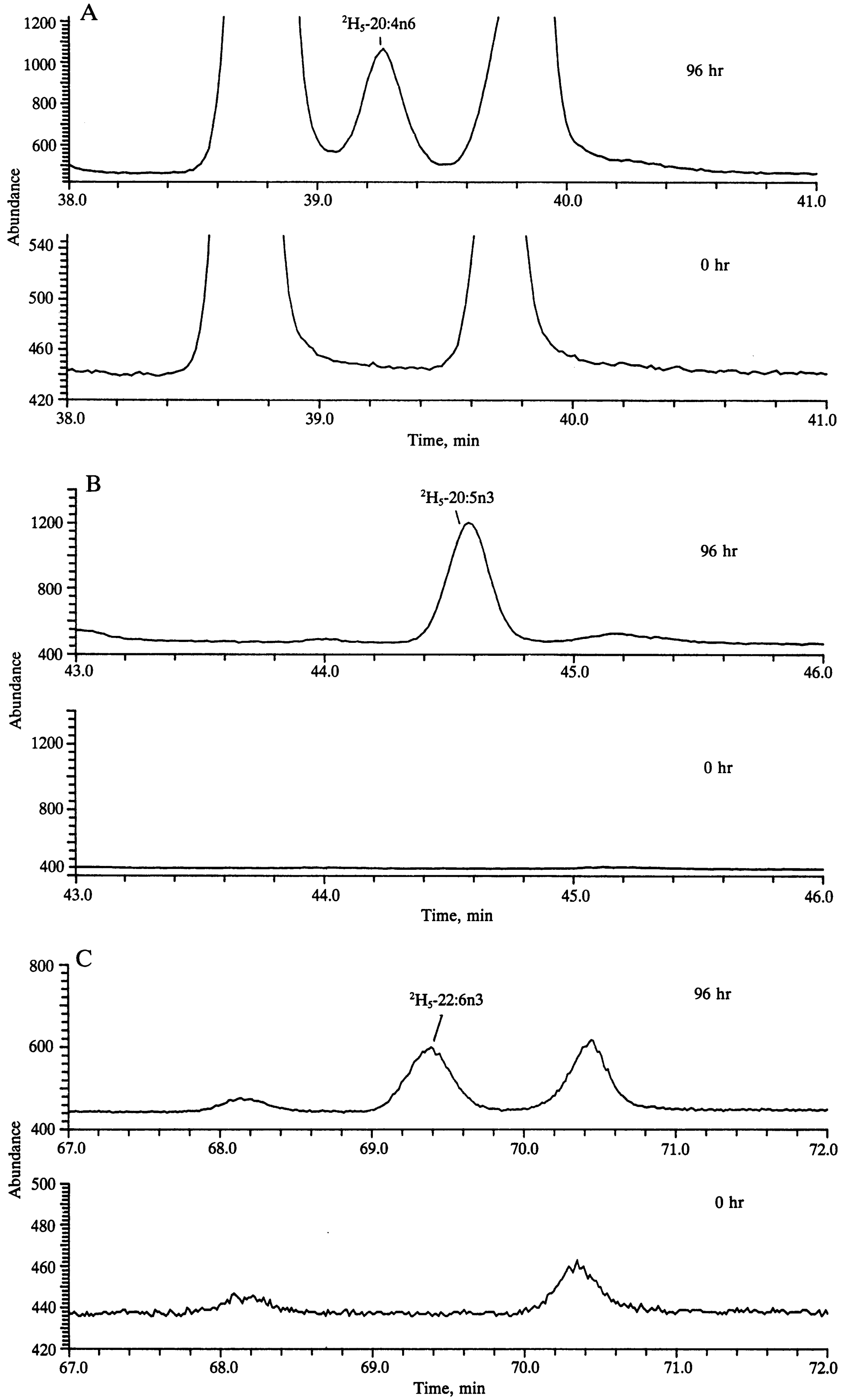

FIG. 2. Selected ion chromatograms of long-chain polyunsaturated fatty acids formed from 18-carbon essential fatty acids. Examples of selected responses from subject s3 at the time points indicated after a single dose of $^2H_5$-18:2n6 and $^2H_5$-18:3n3. The responses for each fatty acid metabolite are shown after 0 hr, a time at which little or no product has been formed, and after 96 hr, showing a positive response. (*A*) The single ion tracing at *m/z* 308—i.e., the mass of the $^2H_5$-20:4n6—is presented. The peaks at 38.8 and 39.8 min represent natural abundance ions of endogenous 20:3n6 (M+3) and 20:4n6 (M+5), respectively. (*B*) The single ion tracing at *m/z* at 306—i.e., the mass of $^2H_5$-20:5n3—is shown. (*C*) The single ion tracing at *m/z* 332—i.e., the mass of $^2H_5$-22:6n3—is shown. The peaks at 68.1 and 70.4 min represent natural abundance ions of 22:5n3 (M+3) and 22:6n3 (M+5), respectively.

taken into account. Also, the amounts of the precursors are underestimated due to limited blood sampling points during their rising phase within the first 24 hr. Finally, it must be noted that these infants were all hospitalized for treatment of various ailments (Table 1) and this may have had a depressant effect on fatty acid biosynthetic rates.

It appeared that the levels of $^2H_5$-18:2n6 were greater than those of $^2H_5$-18:3n3 in the plasma during the first 24 hr (Fig. 1). The $^2H_5$-18:2n6 appears to have been better absorbed than the $^2H_5$-18:3n3; however, an alternative explanation for this may be the greater rate of oxidative degradation of 18:3n3 to CO_2 reported by others (24). It should also be noted that the conversion of $^2H_5$-18:3n3 to $^2H_5$-20:5n3 occurs earlier and the percentage of deuterated precursor conversion is greater than for the corresponding enzymatic steps for $^2H_5$-18:2n6.

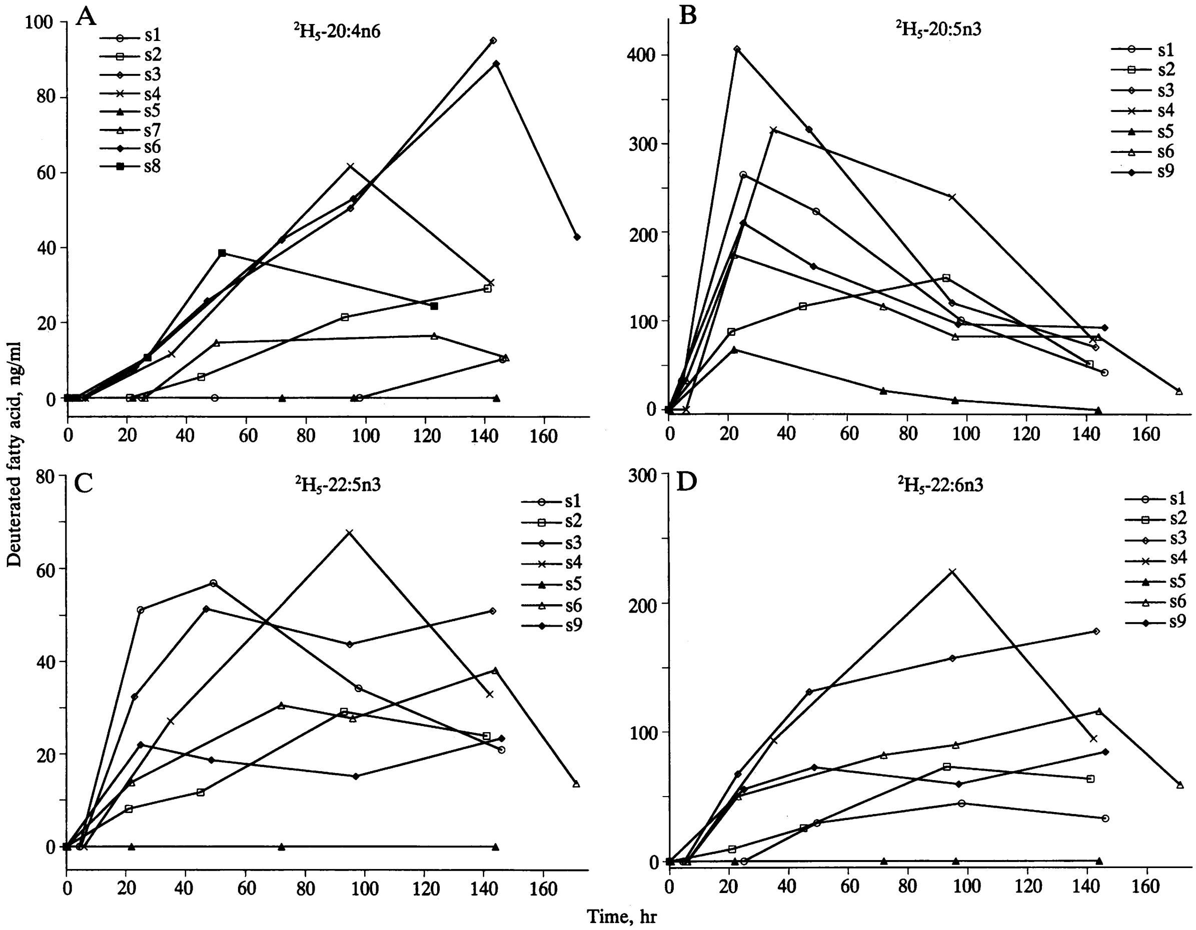

FIG. 3. Time course of deuterated arachidonic, eicosapentaenoic, docosapentaenoic, and docosahexaenoic acid appearance and decay in human infant plasma *in vivo.* Data represent the absolute quantities of the deuterated fatty acid indicated in ng/ml of plasma. (*A*) $^2H_5$-20:4n6. (*B*) $^2H_5$-20:5n3. (*C*) $^2H_5$-22:5n3. (*D*) $^2H_5$-22:6n3.

Our observations indicate that infants as small as 2 kg (and 32 weeks gestational age) are capable of elongation and desaturation of essential fatty acids and, in particular, are capable of forming plasma 20:4n6 and 22:6n3. These findings have implications for the question of their dietary fat requirements. Recent autopsy studies by Makrides *et al.* (25) and by Farquharson *et al.* (26) of infants fed infant formula or mother's milk have shown a decrease in brain 22:6n3 in those fed formulas devoid of 22:6n3 but containing 18:3n3. A large body of studies now exists which suggests that the decrease in brain 22:6n3 associated with vegetable oil-based formulas leads to suboptimal neural development and performance (3–11, 27–33). Apparently the rate of 22:6n3 formation from 18:3n3 is inadequate at this early stage of life. Additional studies are needed to define the optimal manner in which long-chain polyunsaturated fatty acid requirements of artificially fed human infants may be met.

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