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Phospholipid Metabolism during the Development of the Liver. The Incorporation of [1,2-^{14}C]Ethanolamine, [2-^{3}H]*myo*-Inositol and L-[U-^{14}C]Serine into Phospholipids by Liver Slices*

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ABSTRACT: The incorporation of [1,2-^{14}C]ethanolamine, L-[U-^{14}C]serine, and [2-^{3}H]*myo*-inositol into phospholipids has been determined with liver slices from fetal, young, and adult rats.

The rate of incorporation was determined with the concentration of precursor that gave a maximal amount of incorporation and gave a linear rate of incorporation for at least 1 hr. [1,2-^{14}C]Ethanolamine and [2-^{3}H]*myo*-inositol were incorporated intact into ethanolamine phosphoglyceride and inositol phosphoglyceride, respectively. The radioactivity from L-[U-^{14}C]serine was incorporated into all parts of the phospholipid molecule (glycerol, fatty acids, and bases). The amount of incorporation into each part was determined. The ability of liver slices to incorporate [1,2-^{14}C]ethanolamine into ethanolamine phosphoglycerides was low in −5-day fetal (20% of adult), increased to 60% of the adult before birth, and continued to increase to 80% of the adult by 1 day after birth. The rate of incorporation increased again between 5 and 6 days. The incorporation of [2-^{3}H]*myo*-inositol was also low in −5-day fetal and increased to 40% of the adult before birth. The developmental pattern was very similar to that observed for the incorporation of [1,2-^{14}C]ethanolamine. The incorporation of L-[U-^{14}C]serine was about 30% of the adult in −4-day fetal and increased continually until adult levels were reached at 4 days after birth. The incorporation of radioactivity from L-[U-^{14}C]serine into fatty acids, glycerol, and cholesterol was higher in the fetal liver than in adult liver. The kinetics of incorporation of [1,2-^{14}C]ethanolamine into phosphorylethanolamine and ethanolamine phosphoglycerides indicated that the developmental changes in the pathway occurred beyond the phosphorylation of ethanolamine.

The ultrastructure of hepatocytes and their organelles undergoes a regular sequence of changes during embryonic (Dadoune, 1963; Wood, 1967) and neonatal development (Stempak, 1967). The amount of smooth endoplasmic reticulum increases significantly during the period from 1 to 2 days before birth to 2–3 days after birth (Dallner *et al.*, 1966). Phospholipids, a major component of these membrane systems, increase in concentration in the liver immediately following birth (Weinhold and Villee, 1965). These observations suggest that the ability of hepatocytes to synthesize and modify the individual phospholipids changes during development. The investigation of these changes should contribute to an understanding of the mechanisms that control phospholipid synthesis as well as the formation and alteration of membranes.

We have previously reported the results from studies on the biosynthesis of choline phosphoglycerides by liver slices from fetal, newborn, and adult rats (Weinhold, 1969). In

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these studies, we found that both the direct pathway, choline incorporation *via* CDP-choline (Kennedy, 1961), and the pathway that involves the methylation of phosphatidylethanolamine (Bremer *et al.*, 1960) change during prenatal and postnatal development. Artom (1969) has reported that the ability of liver homogenates to carry out the methylation of endogenous phosphatidylethanolamine by [*methyl*-^{14}C]*S*-adenosylmethionine is low in fetal liver and increases immediately after birth. Baldwin and Cornatzer (1968) have measured in the liver from developing rabbits the activity of CDP-choline diglyceride transferase and the methylation of phosphatidyl-*N*,*N*-dimethylethanolamine by *S*-adenosylmethionine. Both were found to be low in the prenatal animals and began to increase shortly before birth.

Studies on the synthesis of ethanolamine phosphoglycerides, serine phosphoglycerides, and inositol phosphoglycerides during liver development have not been reported. This report describes the changes during development in the ability of liver slices to incorporate [1,2-^{14}C]ethanolamine, L-[U-^{14}C]serine, and [2-^{3}H]*myo*-inositol into phospholipids.

Materials and Methods

Pregnant rats of specified delivery dates (±12 hr) and adult controls (180–220 g) were purchased from Holtzman Co., Madison, Wis. All adult controls were females. [1,2-^{14}C]-Ethanolamine, [2-^{3}H]*myo*-inositol, and L-[U-^{14}C]serine were obtained from New England Nuclear Corp.

All animals had free access to a standard commercial diet and were sacrificed between 8:00 and 10:00 AM. Liver slices were prepared with a Stadie-Riggs microtome as previously described (Weinhold and Villee, 1965). Slices (200–250 mg wet weight) were suspended in 2.0 ml of Krebs bicarbonate medium, pH 7.4 (Krebs and Henseleit, 1932), that contained 2 mg/ml of glucose and 1 μCi/ml of radioactivity. Incubations were performed at 37° under 95% O_2–5% CO_2. The specific activity of the ^{14}C labeled precursors was diluted so that the addition of 1 μCi of radioactivity/ml of media gave the desired concentration. The specific activity of the [2-^{3}H]*myo*-inositol was diluted so that the addition of 5 μCi/ml of media gave the desired concentration. The slices were removed from the incubation medium and rinsed with cold isotonic saline, and the lipids extracted by the procedure of Folch (Folch *et al.*, 1957) as modified previously (Weinhold, 1968). Thin-layer chromatography of the phospholipids was performed essentially as described by Skipski *et al.* (1964). Two-dimensional thin-layer chromatography was performed with the solvent systems chloroform–methanol–acetic acid–water (65:25:8:4, v/v) in the first direction and chloroform–methanol–concentrated NH_4OH–water (65:35:5:2.5, v/v) in the second direction. Two-dimensional chromatography on silica gel impregnated paper was done as described by Wuthier (1966). Thin-layer chromatography of the neutral lipids was performed on silica gel H plates with the solvent system described by Malins and Mangold (1960). Methanolysis of the total lipids was done according to the method described by Tarlov and Kennedy (1965). The water-soluble products from the methanolysis were separated by thin-layer chromatography on precoated cellulose plates type (Q2E) that were manufactured by Quantum Industries, Fairfield, N. J. The chloroform-soluble products after methanolysis of the total lipid were separated in the same system that was used for the separation of the neutral lipids.

Phosphorylethanolamine was separated and analyzed by the same general method that was described previously for the analysis of phosphorylcholine (Weinhold, 1968).

Total hydrolysis of the lipid was achieved in 6 N HCl at 110° for 8 hr in sealed tubes. The fatty acids were separated from the hydrolysate by three extractions with equal volumes of hexane. The water-soluble products were separated by thin-layer chromatography on silica gel H plates with the solvent system methanol–concentrated HCl (95:5, v/v).

Radioactivity was determined in a liquid scintillation spectrometer. The counts were corrected to 100% counting efficiency by the channels ratio method (Bush, 1963). Samples that contained water were counted in Bray's scintillation fluid (Bray, 1960). Generally, the amount of radioactivity in the various phospholipids after separation by thin-layer chromatography was determined by scraping the different areas on the silica gel directly into scintillation vials and counting the radioactivity after the addition of the usual toluene scintillation fluid. If it was necessary to recover the lipids, the silica gel areas were scraped into tubes and the lipid was extracted as previously described (Weinhold and Villee, 1965).

Results

Incorporation of [1,2-^{14}C]Ethanolamine. The amount of incorporation of ethanolamine into total tissue lipid after a 60-min incubation was determined at different concentrations of ethanolamine in the incubation medium. With fetal (−2 day), 1-day-old young, and adult rats, maximum incorporation was obtained at a concentration of 0.6 mM ethanolamine. The concentration of ethanolamine routinely employed in all subsequent experiments was 1.3 mM. The incorporation of [1,2-^{14}C]ethanolamine at a concentration of 1.3 mM was linear for 1 hr for slices from fetal, young, and adult livers.

Liver slices from −2-day fetal, 2-hr newborn, and adult rats were incubated with [1,2-^{14}C]ethanolamine for 1 hr. Portions of the total lipid extract from each incubation were analyzed by several methods. These included: one-dimensional thin-layer chromatography, two-dimensional thin-layer chromatography, and two-dimensional chromatography on silica gel impregnated paper. All of the above methods indicated that 90–93% of the radioactivity was associated with the ethanolamine phosphoglycerides. Samples of the total lipid were submitted to mild alkaline hydrolysis in methanol. This procedure converted 95–97% of the radioactivity in the lipid from fetal incubation and 99% of the radioactivity in adult lipid into water-soluble products. Chromatographic separation of the water-soluble products indicated that all of the radioactivity migrated similarly to *sn*-glycero-3-phosphorylethanolamine. Complete acid hydrolysis of the total lipids liberated 97–99% of the radioactivity as water-soluble products. Thin-layer chromatography of the water-soluble products indicated that 98% of the radioactivity migrated similarly to ethanolamine. There was a very small amount of radioactivity that moved like choline (0.6%). The results from the above series of analysis indicate that [1,2-^{14}C]ethanolamine is specifically incorporated into ethanolamine phosphogyleerides and that the radioactivity in the ethanol phosphoglycerides is associated with the ethanolamine portion of the molecule.

The changes during development in the ability of liver slices to incorporate [1,2-^{14}C]ethanolamine into ethanolamine phosphoglycerides are shown in Figure 1. The incorporation is low in fetal liver at −5, −4, and −3 days and begins to

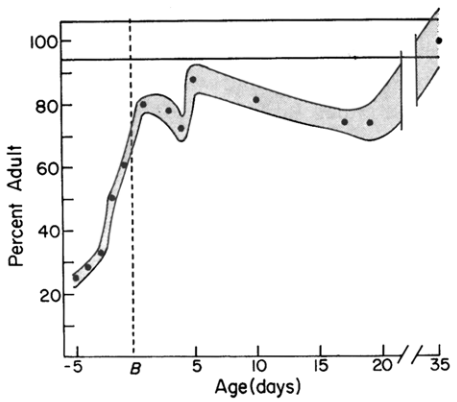

FIGURE 1: The incorporation of [1,2-^{14}C]ethanolamine into ethanolamine phosphoglycerides by liver slices from developing rats. Liver slices were incubated for 1 hr. All results were expressed on a wet weight basis. Each point is the average of at least four separate experiments. An experiment consisted of triplicate incubations of slices that were prepared from the livers from all the fetuses from a pregnant rat or from the livers of four pups from a single litter. Adult rats were measured with each experiment, and these results were averaged to obtain the adult value of 1598 cpm/mg wet tissue. The width of the line indicates ±1 SE. The solid line above and below the 100% indicates ±1 SE for the adult.

increase toward adult levels at −2 days. The incorporation by young rats, age 1 to 4 days, is about 80% of the adult level. An increase to 90% of the adult values occurs at 5 days; however, adult levels are not completely reached until after 20 days.

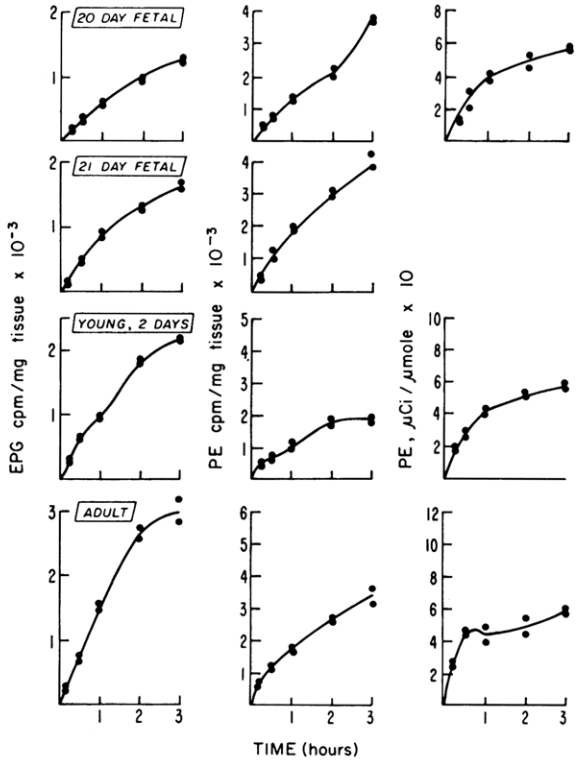

FIGURE 2: The rate of incorporation of [1,2-^{14}C]ethanolamine into phosphorylethanolamine and the ethanolamine phosphoglycerides by liver slices. Each point is the result from a single incubation. EPG and PE refer to ethanolamine phosphoglycerides and phosphorylethanolamine, respectively.

TABLE I: Estimation of the Activity of the Reactions from Phosphorylethanolamine (PE) to Ethanolamine Phosphoglycerides (EPG).[a]

Age	EPG (μCi/hr g of Tissue)	PE (μCi/μmole)	EPG (μmoles/hr g of Tissue)
−2 day	0.148	0.428	0.345
+2 day	0.359	0.429	0.837
Adult	0.522	0.453	1.152

[a] The total incorporation of radioactivity into ethanolamine phosphoglycerides is the amount of incorporation that occurred during the second hour of incubation. The specific activity of phosphorylethanolamine is the average between 1- and 2-hr incubation.

A series of experiments were performed where liver slices from fetal, young, and adult rats were incubated with [1,2-^{14}C]-ethanolamine for various lengths of time. The incorporation of radioactivity into phosphorylethanolamine and ethanolamine phosphoglycerides was determined. The rate of incorporation of [1,2-^{14}C]ethanolamine into ethanolamine phosphoglycerides is linear for the first hour in all experiments (Figure 2). The rate begins to fall at 2 hr for the −2- and −1-day fetal, but does not begin to drop in young rat liver until after 2 hr. There is a definite difference in the incorporation of [1,2-^{14}C]ethanolamine into phosphorylethanolamine between −2-day fetal and 2-day-old young. In −2-day the amount of radioactivity in phosphorylethanolamine continues to increase with time, whereas in 2-day-old rats the radioactivity levels off after 2-hr incubation. However, the specific activity curves for phosphorylethanolamine are similar in all cases. The incorporation of phosphorylethanolamine into ethanolamine phosphoglycerides during the period between 1-hr incubation and 2-hr incubation can be estimated for the experiments shown in Figure 2. For example, the total incorporation of radioactivity into ethanolamine phosphoglycerides by adult liver slices at 1-hr incubation and at 2-hr incubation is 0.692 and 1.212 μCi per g, respectively, which gives an increase of 0.522 μCi/g for the period between 1 and 2 hr. The specific activity of phosphorylethanolamine after 1-hr and after 2-hr incubation is 0.426 and 0.479 μCi per μmole, respectively. The average specific activity during the 1-hr period would be 0.453 μCi/μmole. Therefore, the total incorporation of phosphorylethanolamine into ethanolamine phosphoglyceride during that 1-hr period can be estimated by dividing the amount of radioactivity incorporated (0.522 μCi/g) by the average specific activity of the phosphorylethanolamine (0.453 μCi/μmole). This gives a value of 1.152 μmoles/g. Similar calculations have been done for the incubations with −2-day fetal and +2-day young. The results, shown in Table I, indicate that the rate of incorporation of phosphorylethanolamine into ethanolamine phosphoglycerides in −2-day fetal is about one-third that in the adult liver. The rate of incorporation in the young animals is higher than the fetal but has not yet reached the adult rate. These calculations assume that all of the radioactivity incorporated into the ethanolamine phosphoglycerides occurs *via* phosphorylethanolamine and that the direct exchange of [1,2-^{14}C]-

TABLE II: Incorporation of L-[U-^{14}C]Serine into Lipid by Liver Slices.[a]

Age (Day)	No. of Exp	Total Lipid	SPG[c]	Neutral Lipid	Total Fatty Acids	Cholesterol
Fetal						
−3	5	557 ± 8	93 ± 9	317 ± 7	219 ± 5	126 ± 6
−2	3	424 ± 16	105 ± 1	199 ± 6	144 ± 17	80 ± 9
−1	3	324 ± 9	122 ± 1	107 ± 7	87 ± 7	29 ± 1
−0.5	3	373 ± 5	142 ± 3	81 ± 6	95 ± 6	29 ± 2
Young						
2	3	420 ± 27	182 ± 27	47 ± 1	32 ± 4	ND[b]
3	3	397 ± 19	239 ± 22	36 ± 2	21 ± 1	ND
4	1	460	253	45	36	ND
Adult						
	11	455 ± 50	288 ± 18	90 ± 20	106 ± 32	4 ± 0.7

[a] The values are represented as counts per minute per milligram of fresh tissue plus and/or minus standard error of the mean. The neutral lipids were those that migrated at or near the solvent front in the thin-layer chromatography of the phospholipids. The fatty acid (methyl esters + free fatty acid) and cholesterol were obtained by thin-layer chromatography of the chloroform phase obtained after methanolysis. [b] Not detectable. [c] SPG = serine phosphoglycerides.

ethanolamine into ethanolamine phosphoglycerides is not significant (see Discussion).

Incorporation of [2-^{3}H]myo-Inositol. Initial experiments with −2-day fetal and adult liver slices indicated that the incorporation of [2-^{3}H]*myo*-inositol into inositol phosphoglycerides was linear for 2 hr at an inositol concentration of 1.0 mM in the incubation medium. Analysis of the total lipid extract by thin-layer chromatography and by mild alkaline hydrolysis in methanol showed that for both the fetal and adult slices all of the radioactivity was located in the inositol phosphoglycerides.

Slices from fetal liver at −4 to −2-days of age incorporated [2-^{3}H]*myo*-inositol into inositol phosphoglycerides at a rate of about 16% of the adult rate (Figure 3). The incorporation increases to 26% at −1-day and to about 40% just prior to birth. There is little change in the incorporation in young animals until the age of 5 days, when another increase in activity is observed. Adult levels are slowly approached over the next 25 days.

Incorporation of L-[U-^{14}C]Serine. Incubations were performed with L-[U-^{14}C]serine at a concentration in the medium of 1.0 mM. A constant rate of incorporation of radioactivity into total lipid was obtained for 1–1.5 hr with all ages of animals. The standard incubation time was 1 hr. The incorporation into total lipid was highest in the −4-day fetal and declined slightly in −3- and −2-day fetal rats (Table II). The incorporation in −4-day fetal was slightly higher than in the adult, while the incorporation in −1-day fetal was slightly lower than in the adult. The incorporation into the serine phosphoglycerides, separated by thin layer chromatography, was considerably lower in −4-day fetal liver than in adult, and increased as gestation increased. Adult levels were approached at about 4 days after birth. The incorporation of radioactivity from L-[U-^{14}C]serine into neutral lipids was considerably higher in −4-day fetal than in adult and declined as gestation progressed. Fetal liver incorporates much more radioactivity from L-[U-^{14}C]serine into fatty acids and cholesterol than does adult liver. This incorporation also declines as gestation progresses and coincides with the pattern of incorporation into the neutral lipid fraction. Fetal liver at −4 days incorporates into fatty acids an amount of radioactivity about twice that found for the adult, whereas the incorporation into cholesterol is 30 times higher in −4-day fetal than in adult. The incorporation into fatty acids is lower in young animals than in the adult.

A portion of the total lipid extract from each incubation was also analyzed by the methanolysis procedure. The incorporation of radioactivity into the serine phosphoglycerides, determined by this procedure, was the same as that found previously by thin-layer chromatography of the intact phospholipids (Table III). Therefore, there were insignificant amounts of radioactivity in the fatty acids of the serine phosphoglycerides. The incorporation of radioactivity from L-[U-^{14}C]serine into *sn*-glycero-3-phosphorylethanolamine and *sn*-glycero-3-phosphorylcholine was higher in fetal and young than in adult. This may be due to higher incorporation of radioactivity into glycerol in the fetal and young (see Table IV).

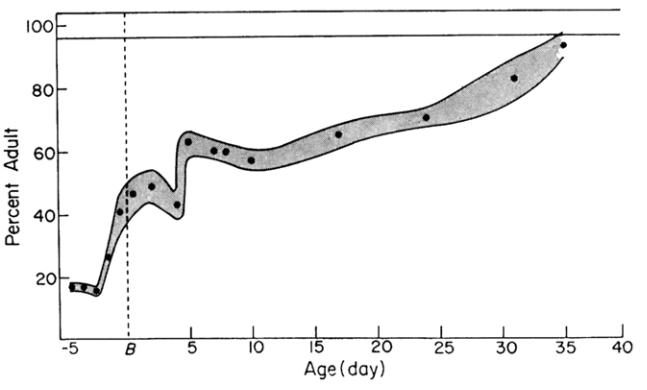

FIGURE 3: The incorporation of [2-^{3}H]*myo*-inositol into inositol phosphoglycerides by liver slices from developing rats. See Figure 1 for the experimental details. The average adult value was 3187 cpm/mg wet tissue.

TABLE III: Incorporation of L-[U-^{14}C]Serine into *sn*-Glycero-3-phosphorylcholine (GPC), *sn*-Glycero-3-phosphorylethanolamine (GPE), and *sn*-Glycero-3-phosphorylserine (GPS).[a]

Age	Counts/min per mg of Fresh Tissue		
	GPS	GPE	GPC
Fetal			
−3 (4)	75 ± 5	33 ± 3	15 ± 2
−2 (3)	80 ± 10	33 ± 2	17 ± 3
−1 (3)	114 ± 9	24 ± 4	17 ± 2
−0.5 (3)	124 ± 1	28 ± 3	20 ± 1
Young			
2 (3)	223 ± 31	57 ± 4	41 ± 4
3 (3)	249 ± 27	37 ± 2	23 ± 2
4 (1)	258	60	45
Adult			
(3)	271 ± 31	14 ± 1	8 ± 1

[a] The phosphate esters were obtained by alkaline alcoholysis of the total lipids that were extracted from slices previously incubated with L-[U-^{14}C]serine. The phosphate esters were separated by thin-layer chromatography on cellulose (see Methods). Each value is the average plus and/or minus standard error of the mean of the results from the number of separate experiments shown in parentheses.

TABLE IV: Incorporation of L-[U-^{14}C]Serine into Water-Soluble Products from the Acid Hydrolysis of the Total Lipid.

Age	Counts/min per mg of Tissue			
	Serine	Ethanolamine	Choline	Glycerol
Fetal				
−1	130	15	3	178
−0.5	111	14	3	161
Young				
16 days	250	18	3	72
Adult	352	23	66	49

Portions of the total lipid from some of the incubations were hydrolyzed completely with 6 N HCl and the water-soluble products separated by paper chromatography. There is appreciable incorporation of radioactivity into the glycerol portion of the lipid, particularly in the fetal liver slices (Table IV). Nevertheless, the incorporation into the serine phosphoglycerides, when determined from the hydrolysis data, is very similar to those obtained both by thin-layer chromatography and by methanolysis. In addition, a portion of the lipid from incubations of −4-day fetal, −3-day fetal, and adult liver slices was subjected to methanolysis and the *sn*-glycero-3-phosphorylserine isolated by thin-layer chromatography. The fraction was subsequently hydrolyzed in 6 N HCl and the water-soluble products separated by paper chromatography. In all samples, the amount of radioactivity in the glycerol portion of the *sn*-glycero-3-phosphorylserine was less than 10% of the total radioactivity in the molecule.

Discussion

Both [1,2-^{14}C]ethanolamine and [2-^{3}H]*myo*-inositol were incorporated specifically into ethanolamine phosphoglycerides and inositol phosphoglycerides, respectively. We assume that ethanolamine was incorporated *via* CDP-ethanolamine as described by Kennedy and Weiss (1956), and that inositol was incorporated by the series of reactions previously reported to occur in liver (Paulus and Kennedy, 1959). The rapid incorporation of [1,2-^{14}C]ethanolamine into phosphorylethanolamine and the attainment of a high specific activity in the phosphorylethanolamine support this assumption about ethanolamine incorporation. However, the possibility that some of the radioactivity may have been incorporated by another process, such as exchange, cannot be completely discarded. Evidence has been presented that choline can be incorporated into choline phosphoglycerides without the use of the CDP-choline pathway (Treble *et al.*, 1970; Dils and Hubscher, 1961). Some labeled ethanolamine also can apparently be incorporated into ethanolamine phosphoglycerides by processes that do not appear to be energy dependent (Borkenhagen *et al.*, 1961).

The developmental patterns for the incorporation of [1,2-^{14}C]ethanolamine and [2-^{3}H]*myo*-inositol are very similar. In both cases, the incorporation is low in −5- to −3-day fetal livers; the incorporation begins to increase at −2 days and reaches a plateau level after birth. At 5 days, the incorporation again increases and thereafter gradually approaches the adult levels. The levels of incorporation of radioactive precursors, measured 1 day after birth, are in both cases about three times the levels observed in −5-day livers. These results could be caused by some change in the slice preparations that would affect both precursor incorporations similarly. The most obvious factor would be the permeability of the slices to the radioactive precursors in the medium. The results of the timed incubations with [1,2-^{14}C]ethanolamine do not support this possibility. The slices from fetal liver incorporate [1,2-^{14}C]ethanolamine into phosphorylethanolamine to the same or higher extent than do liver slices from 2-day-old and adult animals. The results from previous experiments with [*methyl*-^{14}C]choline and [*methyl*-^{14}C]methionine (Weinhold, 1969) also argue against any developmental change in a common tissue characteristic since the developmental patterns observed for the incorporation of these precursors differ both from each other and from the patterns observed in the present experiments. Therefore, it would seem reasonable to conclude that the metabolic pathways for the incorporation of ethanolamine into ethanolamine phosphoglycerides and inositol into inositol phosphoglycerides develop in parallel and perhaps are associated with some common membrane function.

The kinetics of the incorporation of [1,2-^{14}C]ethanolamine into phosphorylethanolamine and ethanolamine phosphoglycerides indicate that the slices from animals of all ages remove ethanolamine from the medium to about the same extent. Thus, as mentioned previously, the developmental changes in the incorporation are not a result of changes in the ability of slices to transport ethanolamine into the cells. Furthermore, the relationships between the specific activity of phosphorylethanolamine and the rate of incorporation of radioactivity into ethanolamine phosphoglycerides indicate that the

changes in the pathway, which are primarily responsible for the changes observed in the total incorporation, occur beyond the phosphorylation of the ethanolamine. This indirectly implies that the rate-determining step in the pathway is either the formation of CDP-ethanolamine from phosphorylethanolamine and CTP or the formation of ethanolamine phosphoglycerides from CDP-ethanolamine and diglycerides.

When liver slices are incubated with L-[U-^{14}C]serine the radioactivity from the L-[U-^{14}C]serine predictably becomes distributed into all of the tissue lipids. The developmental pattern of incorporation of L-[U-^{14}C]serine into total lipid is obviously a summation of independent pathways for serine metabolism. When the lipid is analyzed in some detail, it becomes apparent that the incorporation of intact L-[U-^{14}C]serine into serine phosphoglycerides is low in −3-day fetal liver and increases as development progresses. At the same time, the incorporation of radioactivity from L-[U-^{14}C]-serine into glycerol, fatty acids, and cholesterol is high in −3-day fetal liver and decreases with development. The similarities between the developmental patterns of incorporation of radioactivity into glycerol, fatty acids, and cholesterol indicate that changes in one of the initial steps in serine catabolism probably occur. The primary route for serine catabolism is the conversion of serine into pyruvate by the enzyme serine dehydratase (L-serine: hydrolyase, EC 4.2.1.13) (Greenberg, 1969). This enzyme is known to respond to a variety of dietary and hormonal alterations in the rat (Jost *et al.*, 1968). The activity of serine dehydratase has been shown to be low in fetal rat liver and to increase after birth toward adult levels (Greengard and Dewey, 1967). This pattern of development is not consistent with our observations of an increased catabolism of serine by liver slices from fetal rats. Studies have been reported on the activities in fetal liver of the pathways involved in serine biosynthesis (Cheung *et al.*, 1968). The phosphorylated pathway for serine biosynthesis (D-3-phosphoglycerate ↔ phosphohydroxypyruvate ↔ phosphoserine ↔ serine) is higher in fetal than in the adult liver in a number of different species and appears to be of major importance in fetal liver. The reversal of this pathway would also serve to convert radioactivity from L-[U-^{14}C]serine into glycolytic intermediates and ultimately into glycerol, fatty acids, and cholesterol. The higher activity of this pathway in fetal liver could account for the high apparent catabolism of L-[U-^{14}C]serine observed in our studies.

The activities during liver development in the rat of five separate pathways for the synthesis of phospholipids have been assessed by determining the ability of liver slices to incorporate specific radioactive precursors into phospholipid. These have included the previously published investigations on the incorporation of [*methyl*-^{14}C]choline and L-[*methyl*-^{14}C]methionine into choline phosphoglycerides (Weinhold, 1969), and the present study on the incorporation of [1,2-^{14}C]-ethanolamine, L-[U-^{14}C]serine, and [2-^{3}H]*myo*-inositol into their corresponding phospholipids. All of the pathways begin to increase in activity sometime during the period between 2 days before birth to 1 day after birth. However, the exact time when the activities begin to increase and the relationship of the fetal activity to the adult activity are different for each pathway. The CDP-choline pathway for the synthesis of choline phosphoglycerides is near adult activity in the fetal rat liver and does not increase until after birth, while the methylation pathway for the synthesis of choline phosphoglycerides is considerably lower in fetal liver than in adult liver. The methylation pathway, similar to the CDP-choline pathway, also begins to increase within 24 hr after birth. Both pathways reach activities higher than the adult by 10 days after birth. In contrast, the pathways for the synthesis of ethanolamine phosphoglycerides, inositol phosphoglycerides, and serine phosphoglycerides are all between 20 and 40% of the adult in fetal liver from rats −5-days old and begin to increase 2 to 3 days before birth. Birth does not cause any change in the rate of development of the activities of these pathways. The general increase in the apparent biosynthesis of all of the major types of phospholipids during the late prenatal and early postnatal period of development is consistent with the increase in phospholipid concentration that occurs after birth (Weinhold and Villee, 1965) and with the requirement of the liver to produce more intracellular membranes (Dallner *et al.*, 1966). The fact that the developmental patterns of each pathway are different may reflect a requirement for different turnover rates of the various phospholipids for specific membrane functions.

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Incorporation of L-*O*-Ethylthreonine into Chick Muscle Protein*

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ABSTRACT: While investigating the physiological disposition of the new drug L-*O*-ethyl-^{14}C-threonine in chicks, a high level of radioactivity was observed in muscle and moderate levels in other tissues. The nature of this radioactivity in muscle was further explored by fractionating the muscle tissue into RNA, DNA, lipid, glycogen, and proteins. Most of the radioactivity was found to be associated with proteins. The protein fraction was subjected to intensive investigation to ascertain the nature of the association of the radioactivity with proteins. Results obtained indicated that L-*O*-ethylthreonine was incorporated intact into tissue proteins. This incorporation was demonstrated by studying the reaction products of dansyl chloride with tissue proteins and with protein hydrolysates, and also by fingerprinting the amino acids and polypeptides produced by hydrolytic and enzymatic degradation of muscle protein.

L-*O*-Methylthreonine, an isoleucine antagonist, has been shown to interfere with the incorporation of L-isoleucine into the proteins of Ehrlich ascites carcinoma (Rabinovitz *et al.*, 1955) and into rabbit hemoglobin (Hori and Rabinovitz, 1968), and to inhibit the rate of incorporation of isoleucine into transfer RNA (Smulson and Rabinovitz, 1968). Chick growth studies in our laboratory with the higher homolog L-*O*-ethylthreonine (B. G. Christensen *et al.*, in preparation) indicated that a similar inhibition (G. Olson and W. H. Ott, in preparation) may be occurring between L-*O*-ethylthreonine and isoleucine, *i.e.*, *O*-EtThr appeared to act as an antagonist of isoleucine.

A tracer study of the physiological disposition of L-*O*-ethyl-1-^{14}C-threonine administered orally to chicks revealed that a high level of radioactivity was retained by muscle, with moderate levels residing in other tissues. The results of this study, reported herein, demonstrated that L-*O*-EtThr was incorporated intact into chick muscle proteins. This was done as follows: (1) by studying the distribution of radioactivity retained by muscle tissue among biochemical components such as glycogen, lipid, RNA, DNA, and protein; (2) associating the protein radioactivity with intact *O*-EtThr by electrophoresis and chromatography (fingerprinting) of amino acids liberated after acid hydrolysis; (3) by fingerprinting the radioactivity distribution among polypeptides produced by enzymatic degradation; and (4) by treating the muscle protein with the end-group reagent, dansyl chloride, to demonstrate the absence of a free NH_2 group in the incorporated *O*-EtThr.

Experimental Section

Eight chicks approximately 3 weeks of age, individually housed in electrically heated, metal battery brooders with wire floors, were used for these experiments. Beacon starter broiler mash and water were supplied *ad libitum*. After a 3-day adjustment period, each chick was given a single oral dose of 7.5 mg of L-*O*-ethyl-1-^{14}C-threonine,[1] specific activity $\simeq$0.15 μCi/mg, in a gelatin capsule. The chicks were bled by cardiac puncture and then sacrificed with CO_2 gas at intervals ranging from 6 hr to 6 days. Total excreta collections were made during the experimental period. Immediately after sacrificing, the tissues were harvested, homogenized, and frozen on solid CO_2 until assayed.

Radioactivity Assay. Radioactivity in chick plasma, tissues, and excreta was determined by liquid scintillation counting, using a Packard Tri-Carb scintillation spectrometer Model 3003. Plasma was determined by direct addition of 0.5 ml of plasma to 20 ml of 70:30 scintillator solution. Tissues and excreta were homogenized, aliquots were dried and combusted by the oxygen flask method, and the CO_2-^{14}C produced was dissolved in hyamine for counting in a standard DPO—POPOP scintillator solution in 70:30 toluene–ethanol solvent.

The radioactivity of eluates obtained from papers used for fingerprinting was determined by evaporating them in 3-in. stainless steel planchets, and measuring the activity of the residues in a Sharp Low Beta counter.

Muscle Fractionation. The chick muscle was fractionated into glycogen, RNA, DNA, lipid, and protein, by means of the perchloric acid method developed by Shibko *et al.* (1967). A 20-ml sample of muscle homogenate containing 4.0 g of wet tissue was subjected to the Shibko method without modification. Protein and glycogen samples were assayed radiometrically by the oxygen flask combustion method and liquid scintillation counting. All other samples, including the combined supernatants from glycogen precipitation, were assayed by direct liquid scintillation counting.

Fingerprinting of Muscle Protein Hydrolysate. Approximately 4.0 g of chick muscle processed by the above procedure yielded 894 mg of dried protein. A portion of this protein was subjected to hydrolysis in 6 N HCl at 110°, and the hydrolysate

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[1] Synthesized by our colleague Dr. H. E. Mertel.