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ACTIVITY AND SOME PROPERTIES OF CHOLINE KINASE, CHOLINEPHOSPHATE CYTIDYLTRANSFERASE AND CHOLINE PHOSPHOTRANSFERASE DURING LIVER DEVELOPMENT IN THE RAT

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SUMMARY

The activity of choline kinase (ATP:choline phosphotransferase, EC 2.7.1.32) cholinephosphate cytidyltransferase (CTP:cholinephosphate cytidyltransferase, EC 2.7.7.15) and cholinephosphotransferase (CDP-choline: 1,2-diacylglycerolcholinephosphotransferase, EC 2.7.8.2) was determined during the development of the liver in the rat. Choline kinase activity was located in the $100000 \times g$ supernatant and is slightly lower than adult in –5 day fetal liver. The activity increases to almost 2 times the adult prior to birth and declines to adult levels shortly after birth. The cholinephosphate cytidyltransferase is found at all ages in both the $100000 \times g$ supernatant and in microsomes. The distribution of activity between these two fractions varies with the age of the animal. The activity in the supernatant fraction from –5 day fetal is almost twice the adult values, whereas the activity in the microsome fraction from –5 day fetal does not significantly differ from the adult. The activity in both fractions increases following birth. The activity of cholinephosphotransferase in the presence of exogenous diglyceride was about 10% of the adult values in –5 day fetal liver. The activity increased slightly prior to birth but did not approach the adult levels until 8 days after birth. The addition of diglyceride to the assays did not increase the enzyme activity from fetal liver but caused a 2–3-fold increase in enzyme activity from 5 day and older animals. The developmental activities of the three enzymes are compared to the previously obtained developmental pattern for the incorporation of [$Me-^{14}C$]choline into choline phosphoglycerides by liver slices.

INTRODUCTION

Previous studies on phospholipid metabolism in developing rat liver have shown that the amount of choline phosphoglyceride in fetal liver is about 50% of the adult¹. The ability of liver slices to incorporate $^{32}P_i$ (ref. 1) and [$Me-^{14}C$]choline² into choline phosphoglycerides is also low in the fetal liver and increases towards adult values following birth. The biosynthetic pathway for the *de novo* synthesis of choline phos-

phoglycerides involves the phosphorylation of choline to phosphorylcholine, reaction of the phosphorylcholine with CTP to form CDP-choline, followed by the transfer of phosphorylcholine from CDP-choline to diglyceride to form choline phosphoglycerides³. In this paper we report the results of studies on the activities and properties of each of the three enzymes involved in the pathway during prenatal and postnatal development of the liver.

EXPERIMENTAL

Materials

Pregnant and control female rats were purchased from Holtzman Co., Madison, Wisconsin. The gestational age of the fetal rats was within 12 h as determined by positive sperm on the morning of day one.

[*Me*-¹⁴C]Choline, cytidine diphospho[*Me*-¹⁴C]choline, and [*Me*-¹⁴C]phosphorylcholine were purchased from New England Nuclear Corporation. Cytidine diphosphocholine, phosphorylcholine, phospholipase C (*C. welchii*, Type I) and ATP were obtained from Sigma. Egg choline phosphoglyceride and 1,2-dioleoyl-*sn*-glycerol were purchased from Applied Science Laboratories.

Isolation of choline phosphoglycerides and preparation of 1,2-diacyl-sn-glycerol

Rat liver was extracted with 22 vol. of chloroform-methanol (2:1, v/v) essentially as described by Folch *et al.*⁴. The lipid extract was evaporated *in vacuo* and the lipid residue dissolved in chloroform. Choline phosphoglycerides were isolated from the lipid solution by DEAE-cellulose chromatography according to the method of Rouser *et al.*⁵. The choline phosphoglycerides were hydrolyzed to 1,2-diacyl-*sn*-glycerol with phospholipase C according to the procedure described by Renkonen⁶. The 1,2-diacyl-*sn*-glycerol was purified by preparative thin-layer chromatography on silica gel H with the solvent system toluene-acetone (90:10, v/v). The 1,2-diacyl-*sn*-glycerol was recovered from the silicic acid by extraction with chloroform. The extract was evaporated *in vacuo* and the 1,2-diacyl-*sn*-glycerol dissolved in a small amount of chloroform and stored at -20 °C under nitrogen. This procedure was satisfactory if the diglyceride was not in contact with silicic acid for long periods of time. Because of this problem we have recently used column chromatography on acid-treated florisil to purify the 1,2-diacyl-*sn*-glycerol⁷. The amount of 1,2-diacyl-*sn*-glycerol was determined with the dichromate assay for organic materials described by Amenta⁸ with palmitic acid as a standard or by determining the amount of acyl ester⁹.

Enzyme assays

Choline phosphokinase. A 20% homogenate of liver was prepared in 0.25 M sucrose using a motor-driven teflon pestle homogenizer. The homogenate was centrifuged at 100000 × *g* for 60 min. The supernatant was used for the enzyme assay. Experiments on the subcellular distribution of enzyme activity indicated that all of the activity was located in the 100000 × *g* supernatant in both adult and fetal liver.

Choline kinase (ATP:choline phosphotransferase, EC 2.7.1.32) activity was determined by a modification of the method of McCaman¹⁰. Incubation mixtures contained 0.1 M Tris-HCl, pH 8.0, 10 mM magnesium acetate, 10 mM ATP, and 0.25 mM [*Me*-¹⁴C]choline (0.7 Ci/mole) in a final volume of 1.0 ml. The reaction was

started by the addition of 50 μ l of enzyme that contained 1–2 mg of protein. Incubations were conducted for 20 min at 37 °C and stopped by placing the tubes in boiling water for 2 min. The protein precipitate was removed by centrifugation and 0.5 ml of the supernatant was placed on a column (0.5 cm \times 3 cm) of AG-1 \times 8, 100–200 mesh, OH⁻ form. The column was washed with 10 ml water and the phosphorylcholine was eluted with 0.5 ml of 1.0 M NaOH followed by 1.5 ml of 0.1 M NaOH. The eluate was collected in a liquid scintillation vial, 10 ml of aquasol scintillation fluid (New England Nuclear Corp) was added and the radioactivity was determined in a scintillation spectrometer. Radioactive phosphorylcholine was added to a complete incubation mixture and processed by the column isolation procedure. All of the radioactive phosphorylcholine was recovered while [*Me*-¹⁴C]choline, processed in a similar manner, was not recovered in the NaOH eluate. The reaction rate was constant for 30 min and proportioned to the amount of enzyme if the amount of protein was less than 2.0 mg per incubation.

CTP: cholinephosphate cytidyltransferase. A 20% liver homogenate was prepared in 0.25 M sucrose with a motor-driven teflon pestle homogenizer. The cell debris, nuclei and mitochondria were removed from the homogenate by centrifugation at 10000 \times g for 20 min. The 10000 \times g supernatant was centrifuged at 100000 \times g for 60 min. The microsomal pellet and the supernatant were separated and used for enzyme assays. All assays were performed within 3 h after the animals were sacrificed. In some initial experiments the activity was measured in the 10000 \times g supernatant and the microsome and 100000 \times g supernatant. The sum of the activities in the microsomes and 100000 \times g supernatant was the same as that in the 10000 \times g supernatant.

Cholinephosphate cytidyltransferase (CTP:cholinephosphate cytidyltransferase, EC 2.7.7.15) was assayed by the method of Ansell and Chojnacki¹¹. The volume of the incubation mixture was 0.1 ml and was contained in a disposable polyethylene microcentrifuge tube. The incubation mixture contained 20 mM Tris-malate pH 6.0, 12 mM magnesium acetate, 4.2 mM CTP and 1.6 mM [*Me*-¹⁴C]phosphorylcholine (1000 cpm/ μ mole). The assay was started by the addition of 20 μ l of enzyme preparation and was incubated for 20 min at 37 °C. The reaction was stopped by placing the tubes in boiling water for 2 min. After cooling the mixture with ice, the protein was removed by centrifugation and 50 μ l of the supernatant was applied as a 2-cm streak to Whatman 3 MM chromatography paper. The chromatogram was developed in an ascending direction in isopropanol–20% trichloroacetic acid–16 M ammonia (75:25:0.3, by vol.) to a distance of 15 cm. After the paper was dry, the first 2 cm of each lane were removed, placed in a scintillation fluid, and the radioactivity was determined. A portion of the substrate, [*Me*-¹⁴C]phosphorylcholine, was spotted on paper and counted exactly as the sample. This count was used to calculate the specific activity of the phosphorylcholine. Under these conditions, the rate of CDP-choline production in the assay was linear for 30 min. The production of CDP-choline was proportional to the amount of enzyme preparation if no more than 30 μ l of supernatant was used in the assay.

CDP-choline: 1,2-diglyceride choline phosphotransferase. Microsomes were isolated essentially as described by De Kruffyff *et al.*¹². Cholinephosphotransferase (CDP-choline: 1,2-diglyceride choline phosphotransferase, EC 2.7.8.2) was assayed according to the procedure of Van Golde *et al.*¹³. The reaction mixture contained 4 mM glutathione, 10 mM MgCl₂, 34 mM Tris-HCl pH 7.4, 0.08 mM (0.02 μ Ci)

CDP-[*Me*- ^{14}C]choline, 1.0 mg/ml diglyceride, 0.01 mg/ml Tween 20, and 50 μl of microsomes that were suspended in 0.02 M Tris–0.12 M KCl pH 7.4, at a concentration of 1 mg protein/ml. The reaction was started by the addition of microsomes. The reaction was usually run for 20 min at 37 °C and stopped by the addition of 1.0 ml of chloroform–methanol (2:1, v/v). Blanks were stopped at 0 time. Water (0.2 vol.) was added and the chloroform layer separated from the water–methanol layer by brief centrifugation. The chloroform layer was washed 2 times with Folch upper phase⁴. The washed chloroform solution was added to a scintillation vial and the chloroform evaporated. Toluene scintillation fluid was added and the radioactivity determined in a liquid scintillation spectrometer. Thin-layer chromatography of the chloroform extract showed that all of the radioactivity migrated as choline phosphoglycerides. Diglyceride was prepared as follows. A chloroform solution of diglyceride was added to a small tube and the chloroform evaporated with a stream of nitrogen. The residue was dissolved in acetone and a 0.1 M Tris (pH 7.4)–0.05% Tween 20 solution added so that a final concentration of diglyceride was 5 mg/ml. The acetone was removed under nitrogen at reduced pressure. The resulting suspension of diglyceride was clarified by sonication for 10 min with a Bronson Sonifier Model W185 at a setting of 3.

Analytical methods

Protein was determined by the method of Lowry *et al.*¹⁴.

RESULTS

Choline kinase

Choline kinase is located in the soluble portion of the cell in both fetal and adult liver. The amount of activity in –5 day fetal liver is slightly lower than in adult liver, Fig. 1. The activity increases at –2 day to a peak value that is 320% of the adult

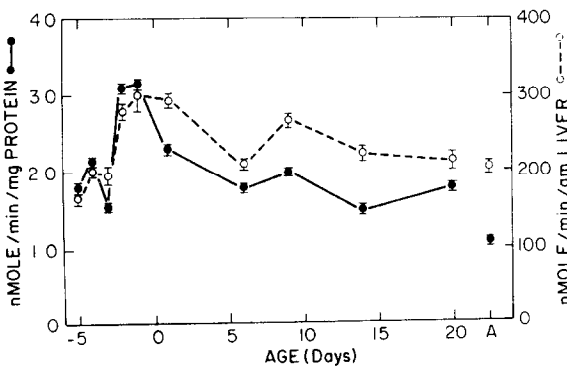


Fig. 1. Choline kinase activity during development of the liver. Each point for fetal and young animals is the average of values obtained from five separate litters. The adult values are averages of 16 separate animals. The bars indicate \pm S.E.

when referred to supernatant protein and 150% of the adult when referred to liver weight. The differences in values is caused by development changes in the amount of

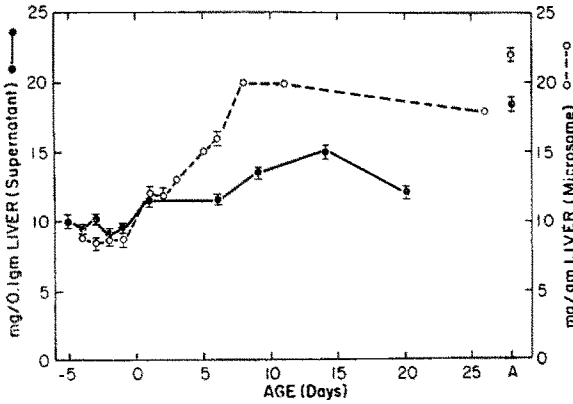


Fig. 2. The developmental changes in the amount of microsomal and cytoplasmic protein in the liver. Each value is the average of the values from 6 or more separate litters or adult animals. The bar represents \pm S.E.

supernatant protein per g liver, Fig. 2. In both cases the activity declines toward adult levels shortly after birth.

CTP: cholinephosphate cytidyltransferase

In both fetal and adult liver cholinephosphate cytidyltransferase activity is found in the $100000 \times g$ supernatant fraction and in the microsomal fraction. No appreciable activity was found in other fractions. Although there is always more activity in the supernatant than in the microsomes, the relative distribution of the activity between these fractions differs at different stages of development, (Fig. 3). In - 5 day fetal there is 7 times more activity in supernatant than in the microsomes and the activity is about twice that found in the adult liver. The activity in the supernatant

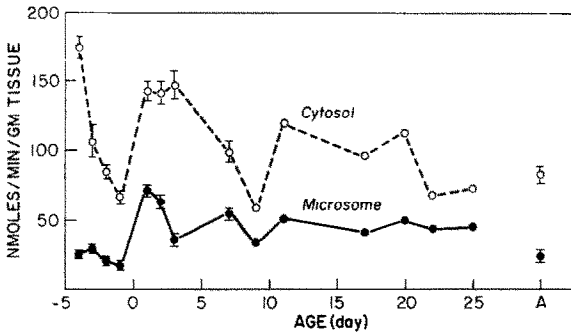


Fig. 3. The activity of CTP:cholinephosphate cytidyltransferase in the $100000 \times g$ supernatant (cytosol) and in microsomes during liver development. Each point where a bar representing \pm S.E. is shown represents the average of values from 4 or more separate litters. The other points represent the average of values from two litters.

drops prior to birth to a value slightly lower than adult and increases after birth to a level about twice the adult level. There appears to be a second decline in activity followed by another peak of activity at around 15 days. This latter rise in activity is less certain because the values are averages of only two experiments. The development-

al pattern of activity in the microsomes is less complex than that of the supernatant. The activity in the microsomes from fetal liver is near the adult level and increases after birth to levels about double the adult at 1 day. Adult levels are essentially reached by 3 days after birth.

Several attempts were made to stimulate the activity in the supernatant by the addition of sonicated suspensions of liver phospholipids. No stimulation was obtained with either fetal or adult enzyme. This is in sharp contrast to our results with developing lung where phospholipid produced a 5–6-fold stimulation of enzyme activity from fetal lung¹⁵.

CDP-choline: diglyceride choline phosphotransferase

Cholinephosphotransferase activity was located entirely in the microsomal fraction in the liver from animals of all ages. Cholinephosphotransferase activity was determined with and without the addition of exogenous diglyceride to the reaction.

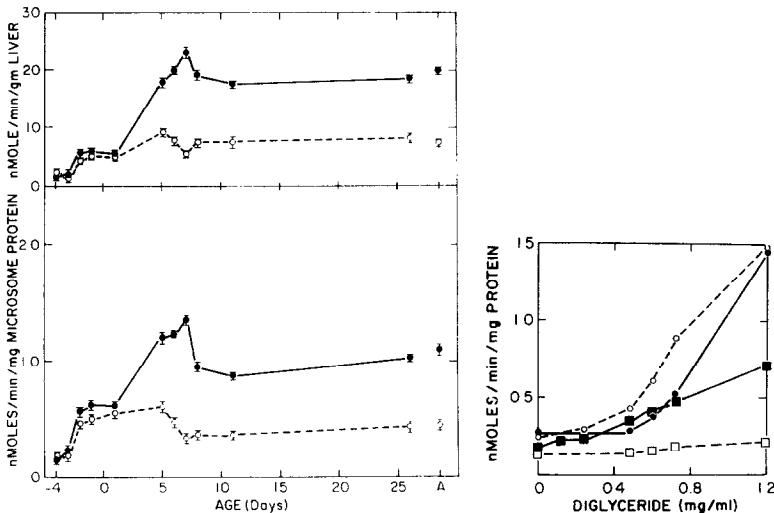


Fig. 4. The activity of CDP-choline:1,2-diglyceride choline phosphotransferase during liver development. Each point is the average of values from 6 separate litters. The adult values are the average from 12 animals. The bar represents \pm S.E. The activity was determined with diglyceride added to the incubation (●—●) and without the addition of diglyceride to the incubation (○—○).

Fig. 5. The effect of diglyceride from adult liver choline phosphoglyceride² and from fetal liver choline phosphoglyceride on the activity of cholinephosphotransferase. The experiment included the determination of activity of enzyme from fetal liver and from adult liver in the presence of either fetal diglyceride or adult diglyceride. Adult enzyme, fetal diglyceride, ○—○—○; adult enzyme, adult diglyceride, ●—●—●; fetal enzyme, fetal diglyceride, ○—○—○; fetal enzyme, adult diglyceride, ●—●—●.

The activity in both cases was low in -4 day liver, Fig. 4. The activity increased from -3 day to -2 days and remained essentially at that value until it increased again between 1 day and 5 days after birth. The amount of activity in the fetal liver and in liver from 1-day-old animals was not increased significantly by the addition of diglyceride. However, in the 5-day and older animals diglyceride produced a 2–3-fold increase in the activity. The activity with or without diglyceride reached the corresponding adult levels between the 1 day and 5 days after birth. A similar pattern was ob-

served when the activity was referred to total microsomal protein or liver weight. The cholinephosphotransferase activity per g of liver measured in the presence of exogenous diglyceride, increases almost 4 fold from -1 day to 8 days. This presumably is a reflection of both the increase in total microsomes per g liver (Fig. 2) and the increase in activity per mg of microsomal protein.

The pronounced difference between fetal and post-natal cholinephosphotransferase in the response to added diglyceride was investigated further. Diglyceride was prepared from choline phosphoglycerides isolated from fetal liver and from adult liver. The ability of these diglyceride preparations to increase the activity of cholinephosphotransferase from both adult and -2 day fetal liver was compared, Fig. 5. The cholinephosphotransferase activity from adult liver was increased by the addition of either fetal diglyceride or adult diglyceride. The cholinephosphotransferase from fetal liver was not significantly affected by the addition of fetal diglyceride. The addition of adult diglyceride gave some increase in activity at higher concentrations.

TABLE I

COMPARISON BETWEEN -4 DAY FETAL AND ADULT OF THE MAXIMAL MEASURED ACTIVITIES OF CHOLINE KINASE, CHOLINEPHOSPHATE CYTIDYLTRANSFERASE AND CHOLINEPHOSPHOTRANSFERASE

	Maximal activity (nmole/min per g liver) ± S.E. (n)	
	-4 day fetal	Adult
Choline kinase	200 ± 10 (5)	205 ± 8 (16)
Cholinephosphate cytidyltransferase*	140 ± 15 (4)	110 ± 10 (9)
Cholinephosphotransferase**	2.0 ± 0.5 (5)	7.3 ± 0.4 (11)

* Sum of supernatant and microsome activity.

** Activity measured with added diglyceride.

The maximal activities of the three enzymes involved in the pathway for the incorporation of choline into choline phosphoglycerides are compared in Table I. The last reaction in the sequence, cholinephosphotransferase, is many fold lower in activity than the other two enzymes. This may or may not reflect what is actually occurring in the tissue under *in vivo* conditions since such things as substrate concentrations, enzyme affinities for the substrates and the influence of inhibitors or stimulators will certainly be factors in determining the actual rate of the pathway. The interpretation is further complicated by the fact that it is difficult to assay the cholinephosphotransferase under conditions that assure maximal activities.

DISCUSSION

The amount of endoplasmic reticulum in the liver hepatocyte increases during development^{16,17}. The most pronounced increase occurs immediately following birth. Earlier studies have shown that the synthesis of choline phosphoglyceride by liver slices increases during the same period of development^{1,2}. The present study has extended these observations by describing the developmental pattern of the three enzymes responsible for the incorporation of choline into choline phosphoglycerides. In Fig. 6 the results are summarized and compared to the developmental pattern of the

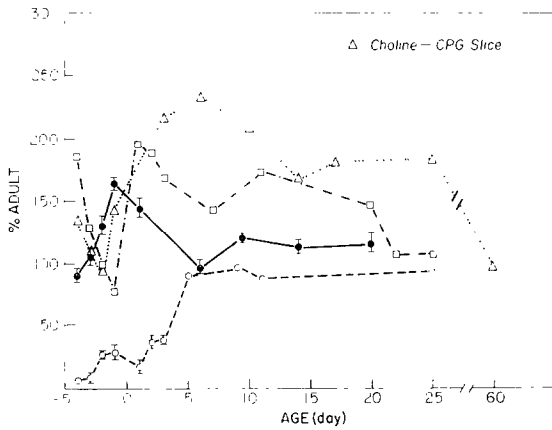


Fig. 6. A comparison of the developmental pattern of activity of choline kinase (●), cholinephosphate cytidyltransferase (□) and cholinephosphotransferase (○) with the activity of slices to incorporate [$Me-^{14}C$]choline into choline phosphoglycerides.

incorporation of [^{14}C]choline into choline phosphoglyceride by liver slices. The developmental pattern of cholinephosphate cytidyltransferase activity coincides rather closely with the developmental pattern of liver slices, particularly in fetal and neonatal period. The low activity of cholinephosphotransferase in fetal liver is difficult to correlate with the slice data. At -4 day the enzyme activity is less than 10% of the adult whereas the incorporation of [^{14}C]choline into choline phosphoglycerides by liver slices is 130% of the adult. Either the assay for cholinephosphotransferase activity does not accurately reflect the true activity of the enzyme or other factors such as substrate and co-factor levels are more significant in determining the rate of the pathway in the slice than are the enzyme capacities. Both cholinephosphate cytidyltransferase and cholinephosphotransferase increase in activity after birth, which coincides with the increase in the formation of endoplasmic reticulum membranes. Both enzyme activities have also been observed to increase together during the increase in choline phosphoglyceride synthesis by barley aleurone cells induced by gibberellin¹⁸ and in cultured lymphocytes after treatment with phytohemagglutinin¹⁹. Baldwin and Cornatzer²⁰ have measured the activity of cholinephosphotransferase in the liver of developing rabbits. They also found that the activity increased following birth to a maximum at about 9 days.

The cholinephosphate cytidyltransferase activity was found at all ages in both the microsome fraction and in the $100000 \times g$ supernatant. This result is in complete agreement with those reported by Wilgram and Kennedy²¹. However, the relative amount of activity in the two subcellular fractions varies with the age of the animal. This is particularly apparent in the fetal liver where the activity in the cytosol is 6 to 7 times greater than in the microsomes. These results suggest that there may be two relatively distinct pools of enzyme, a soluble form of enzyme and a particulate form of the enzyme. Alternatively, if the enzyme is located *in vivo* attached to the membranes in the cell, the stability of this attachment varies with the development of the liver.

The response of cholinephosphotransferase to added diglyceride is quite different with microsome from fetal liver than with microsome from young and adult animals. Diglyceride gives only a slight increase in activity with fetal microsomes. A pos-

sible explanation may be that in young and adult preparation the enzyme is present in amounts greater than the amount of endogenous diglyceride and therefore the activity without added diglyceride is below the maximal activity. Whereas, in fetal microsome there is less enzyme and this amount of enzyme is saturated by the endogenous diglyceride and thus the addition of diglyceride to the incubation does not cause an increase in activity.

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