Enzymes for the Formation of Lecithins by Transmethylation in the Livers of Developing Rats[1] (34359)

CAMILLO ARTOM

Department of Biochemistry, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, North Carolina 27103

Two major mechanisms for the biosynthesis of lecithins (phosphatidyl choline) have been shown to occur: the utilization of intact choline through the intermediate stages of phosphoryl choline and CDP-choline (1), and the "activation" of L-methionine (Me)[2] with formation of *S*-adenosylmethionine (AdMe), followed by the stepwise transfer of methyls to the N of an acceptor phospholipid (2-6).[3]

In a previous investigation (9) the activities of enzymes involved in the former pathway have been determined on the livers, lungs, and brains of developing rats. We have now carried out a similar study of the enzymes responsible for the other pathway, that is: (a) Me-activating enzyme (ATP:L-methionine *S*-adenosyltransferase, EC 2.5.1.6), catalyzing the reaction:

$$\text{Me} + \text{ATP} \rightarrow \text{AdMe} + \text{PP} + \text{P}_i, \quad (1)$$

and (b) phospholipid methyltransferase(s), catalyzing the reactions:

$$\text{phosphatidyl EA} + \text{AdMe} \rightarrow \text{phosphatidyl MEA} + \text{AdH} \quad (2)$$

$$\text{phosphatidyl MEA} + \text{AdMe} \rightarrow \text{phosphatidyl DME} + \text{AdH} \quad (3)$$

$$\text{phosphatidyl DME} + \text{AdMe} \rightarrow \text{lecithin} + \text{AdH} \quad (4)$$

It seems likely that in lower organisms [*e.g.*, Refs. (10-11)], as well as in the mammalian liver [*e.g.*, (12)], reaction (2) is catalyzed by a methyltransferase distinct from the one catalyzing the two subsequent steps. At any rate, while indications have been obtained for the occurrence of the transmethylating pathway in various animal tissues, the liver seems to be the only tissue in which this pathway is quantitatively significant (13-15).

In the present investigation the activities of the two enzymes were determined at various times on the livers of fetuses during the last week of pregnancy, of sucklings, and of youngsters about 30 days old, the values being compared with those obtained on the livers of the mothers, or of adult female rats, sacrificed simultaneously with the fetuses or the offsprings. A short preliminary report has appeared (16).

Experimental Procedure. Female albino rats (Dublin DR., Wistar derived, Dublin Farms, Va.), weighing between 200 and 250 g, were mated, and the day in which a vaginal smear was found to be sperm positive was counted as the first day of pregnancy. The date of pregnancy, thus estimated, agreed within 1 or 2 days with that indicated by the length of the fetuses (17), or with the actual date of birth in those experiments in which

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[2] Abbreviations used: Me, L-methionine; AdMe, L-*S*-adenosylmethionine; EA, ethanolamine; MEA, *N*-methylethanolamine; AdH, *S*-adenosyl homocysteine; DME, *N,N*-dimethylethanolamine; TLC, thin-layer chromatography.

[3] Of the other pathways which have been described, the reacylation of lysolecithins (7) should be regarded as part of a mechanism for the conversion of a lecithin species into another rather than a process for the *de novo* formation of lecithins. The physiological significance of the Ca-activated incorporation of choline into lecithin by liver preparations (8) remains to be ascertained.

pregnancy had not been terminated artificially.

The animals were killed by decapitation and the livers were rapidly excised. Weighed portions of 2–4 pooled livers (fetuses), or of the individual livers (offsprings and adult females), were homogenized partly in 0.03 *M* K-phosphate buffer, pH 6.9 (for the Me-activating enzyme assay), and partly in 0.25 *M* sucrose with 0.002 *M* EDTA (for the methyltransferase assay), a Potter-Elvehjem homogenizer with Teflon pestle being used. After centrifugation at 800*g* for 10 min in a refrigerated centrifuge, the supernatants were further diluted with the respective homogenizing media to a definite volume (mostly 20, or 40 ml/g of fresh tissue) and were used immediately for the enzymatic assays. All operations were carried out in a cold room at about 4°.

Enzymatic assays. For the Me-activating enzyme the method of Mudd *et al.* (18) was used with only minor modifications. The method is based upon the conversion of Me-^{14}C into AdMe-^{14}C which, unlike Me-^{14}C, is retained by Dowex-50–NH_4 and is subsequently eluted with 3 *N* NH_4OH. The radioactivity of the eluate is measured. When ATP or $MgCl_2$ was omitted from the incubation mixture, little or no radioactivity was found in the eluate from the Dowex-50 column. When the complete mixture was used, and the eluate was concentrated and chromatographed on paper with three different solvents, practically all the radioactivity migrated with authentic AdMe.

To assay the phospholipid methyltransferase, the liver homogenate was incubated with AdMe-methyl-^{14}C in Tris–HCl buffer, pH 8.0, as described by Rehbinder and Greenberg (12), except that exogenous phospholipids were not added. At the end of the incubation the lipids were extracted with *n*-butanol, and the radioactivity of the extract was determined. The values, corrected on the basis of the radioactivity of the controls, were assumed to give an approximate estimate of the amounts of methyls transferred from AdMe to phospholipid acceptors present in the liver homogenate. Except for those determinations carried out on materials with very low enzyme activity (most fetal livers), the radioactivity of the controls was usually less than 5% of that of the lipids extracted from the tubes incubated with active liver preparations. Generally, lecithins were the only radioactive compounds clearly demonstrable upon thin-layer chromatography (TLC) of the extracts. Occasionally small amounts of radioactive materials were present in an area corresponding to that of phosphatidyl DME.[4] After hydrolyzing the lipids with boiling 3.5 *N* HCl in methanol and water (2:1) for 3 hr, or with 1 *N* KOH in water and ethanol (9:1) for 16 hr at 38°, at least 90% of the radioacitivity of the water-soluble products could be recovered in the precipitate formed with Reinecke salt. On paper chromatography of the acid hydrolysates, 90% or more of the radioactivity migrated with authentic free choline.

All enzymatic assays were run in duplicate. Moreover, in most cases, two or three different amounts of liver homogenates were used, in order to make sure that the values obtained were falling in the range in which there was at least an approximate proportionality between the amounts of enzyme and those of the products formed. Controls were prepared and treated identically to the experimental samples, except that the enzymes were inactivated before the incubation by boiling (activating enzyme) or by addition of acid (methyltransferase). All tubes were incubated at 37° for 30 min. The experimental values, corrected on the basis of the control values, were expressed as millimicromoles of AdMe synthesized, or of methyls transferred to phospholipids, respectively, per 100 mg of liver protein and per gram of wet tissue.

Analytical methods. Radioactivity was measured in a Packard Tri-Carb scintillation counter with the scintillators in toluene

[4] As reported previously (5), upon TLC with solvent I (see text) phosphatidyl DME migrates well in front of phosphatidyl EA. In contrast, with solvents Iac (chloroform–methanol–acetic acid, 72:25:3, v/v/v), or solvent Ine (chloroform–methanol–water, 70:25:5, v/v/v) which is similar to those used by others (19, 20), the R_f of phosphatidyl DME is slightly lower than that of phosphatidyl EA.

(methyltransferase), or in a thixotropic gel dispersed in xylene–dioxane–ethanol–naphtalene (activating enzyme). Occasionally, as in some of the procedures for the identification of the enzymatic products, a gas-flow GM counter was used. Thin-layer and paper chromatograms were read with a commercial GM radioscanner connected with a ratemeter. In most cases, TLC of the lipid extracts was performed on 50 × 200-mm glass plates, covered with silica gel G and developed with solvent 1 (chloroform–methanol–7 *N* ammonium hydroxide, 60:35:5, v/v/v). Protein was determined according to Lowry *et al.* (21) with crystalline bovine albumin as the standard.

Materials. The Me-methyl-^{14}C and AdMe-methyl-^{14}C were purchased from New England Nuclear Corporation, Boston, Mass., and from International Chemical and Nuclear Corporation, Irvine, Cal., respectively. Paper chromatography of these materials suggested a purity of 98%, or better. Phospholipids, used as standards for TLC, were synthetic products from commercial sources, except for phosphatidyl DME which was the gracious gift of Dr. Eric Baer of the University of Toronto, Canada. Other chemicals and solvents were of reagent grade, except for carrier AdMe (*S*-adenosyl- L-methionine iodide, Calbiochem, Los Angeles, Cal.), which was only 80–90% pure.

Results. Our results are summarized and presented graphically in Fig. 1 and 2. In view of a certain degree of uncertainty in establishing the precise date of the pregnancy (see above) and in an attempt to increase somewhat the significance of the averages, the results obtained from the fetuses on 2 consecutive days were grouped together, each group including the results of 4–8 determinations. Only two determinations on the livers of rats a few hours after birth were made. Each of the remaining values for "sucklings" are the means of 8 or 10 individual determinations. The value for "youngsters" is the mean of the results obtained from rats (of both sexes) killed between days 29 and 36 (28 determinations) and that for "adult" females, is the mean from rats weighing between 200 and 280 g. This group includes 14 mothers, as well as 22 females not pregnant and not nursing. Significant differences were not apparent between the means calculated for the mothers or for the other adult females.

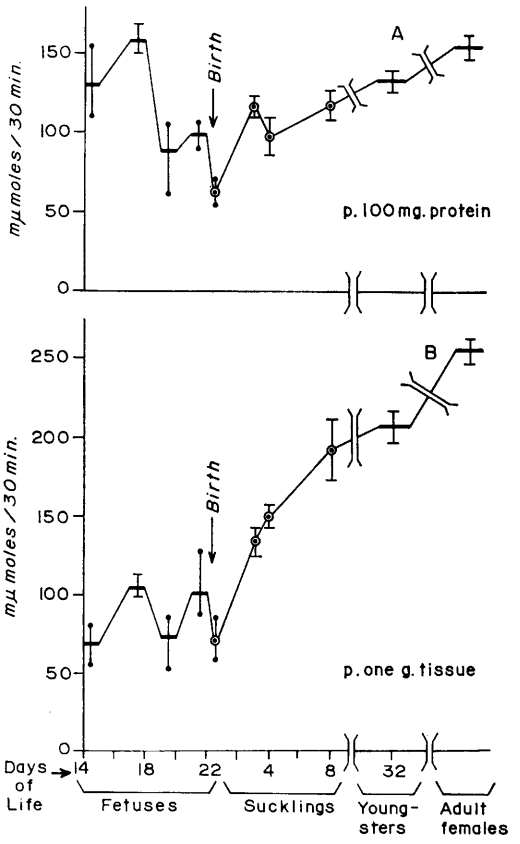

FIG. 1. Developmental pattern of methionine-activating enzyme in rat liver. Values on the curves are the means of those obtained on one (⊙), or on more than one consecutive days (horizontal segments). The ranges (for groups of 6 or less determinations), or the standard errors of the means (for groups of 8 or more determinations) are indicated, respectively, by points, or by bars, above and below the curves. In curve A values have been referred to 100 mg of protein, in curve B to 1 g of moist tissue.

During the last week of fetal life, the mean values for the Me-activating enzyme, expressed as per 100 mg of protein (Fig. 1, curve A), varied betwen 58 and 100% of the mean activity in the livers of adult females (about 65% in the fetuses at term). After a minimum at birth, the activities in-

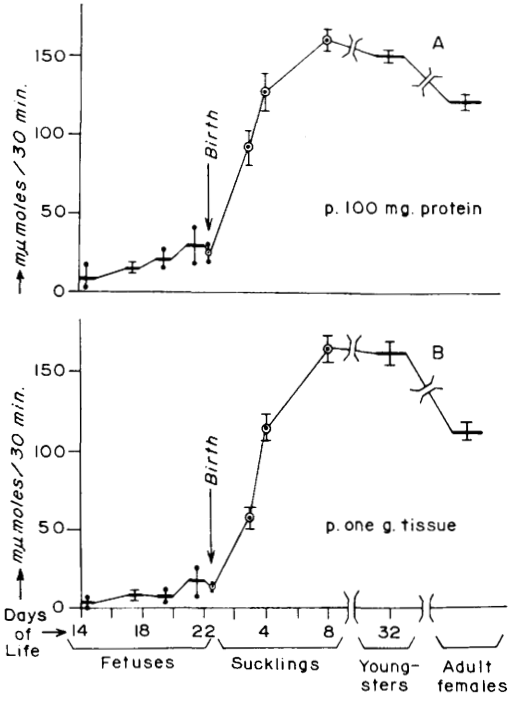

FIG. 2. Developmental pattern of phospholipid methyltransferase in rat liver. For explanations, see legend to Fig. 1.

creased slowly and progressively, reaching their peak in the adult females.

If the results of these determinations are expressed per gram of wet tissue (Fig. 1, curve B), the activities in the fetal livers would represent smaller fractions of the mean activity of the adult livers (27–41%, about 40% at term). Accordingly, the increase after birth looks much more rapid, the adult livers again exhibiting the greatest activity.

In contrast, values for the methyltransferase, whether expressed per 100 mg of protein (Fig. 2, curve A) or per gram of tissue (Fig. 2, curve B), were quite low in the livers of fetuses, even at term. However, immediately after birth the enzymatic activities exhibited a rapid increase with the maximum reached on day 8 after birth and a subsequent moderate decline to the mean adult value.

Discussion. The Me-activating enzyme is already quite active in the fetal livers during the last week of pregnancy. In this respect, it should be pointed out that AdMe is the common methyl donor for a great variety of transmethylation processes. Hence the early development of the capacity of rat fetal livers to synthesize AdMe may well be related to the formation of other methylated compounds and not necessarily to that of lecithins.

After birth the activities of the enzyme seem to increase progressively with peak values reached in the livers of adult females. The disagreement between this and our previous finding of a greater activity of the Me-activating enzyme in the livers of younger than in those of older rats (22) is probably only apparent. Indeed in our previous study both "younger" and "older" rats were males, and it has been shown that in the livers of sexually mature males the enzyme is much less active than in mature adult females (22, 23).

The results of a few determinations of the Me-methyltransferase in the livers of rabbits of various ages were reported very recently by Baldwin and Cornatzer (24). From one of their data it would appear that the enzyme is active 3 days before birth. The discrepancy between this and our present finding of very small activities in the livers of rat fetuses may be due to the difference in animal species, but a difference in the methods for the assay of the enzymatic activity may also be responsible. Indeed those authors used as methyl acceptor, synthetic phosphatidyl DME added as an emulsion to the incubation system, and were actually determining the ability of the tissue to catalyze the last methylating step. In contrast, in the assay we used, only internal methyl acceptors were present and therefore the overall process was followed, including the initial methylating reaction which as mentioned before, is probably catalyzed by a methyl transferase distinct from that catalyzing the two subsequent steps.

In our previous study on enzymes involved in the cytidine-dependent pathway for the synthesis of lecithins from choline (9), it was found that in the liver, brain, and lung of the fetus at term, the activities of choline kinase (EC 2.7.1.32) and cytidyltransferase

(EC 2.7.7.15) were as high, or, more often, higher than in the corresponding tissues of the mothers. Only for the cholinephosphotransferase (EC 2.7.8.2) of liver (and brain) were the values lower in the fetus at term than in the mothers, with the maximum reached a few days after birth and a subsequent moderate decline to the adult level. Some results reported recently by others (25) are in good agreement with these findings. Therefore, the developmental patterns of the choline phosphotransferase and that of the methyltransferase in the livers of rats seem to be similar. However, in the fetus at term the activity of choline phosphotransferase (per unit of protein weight) averaged 66% of the mean activity in the livers of adult females, whereas the mean activity of the methyltransferase in the fetal livers was less than 25% of that in the livers of adult females. (The corresponding values per g of wet tissue would be 33 and 13%). Accordingly, it would appear that in the liver of the rat the cytidine-dependent pathway reaches full development earlier than the transmethylating pathway. Baldwin and Cornatzer (24) who also are reporting the results of some parallel determinations of choline phosphotransferase, are making a similar statement for the liver of rabbit.

The decline in the activity of the methyltransferase in the livers of adult females is in agreement with our previous results in which the activities of the methyltransferase in the livers of young and older males were compared. It may be pointed out that in the livers of sexually mature rats no sex differences in the activities of the methyltransferase could be detected (22).

Finally, it should be emphasized that the present results were obtained with crude homogenates. Only by using purer enzyme preparations will it be possible to state whether, and to what extent, the changes observed are actually due to changes in the amounts of the enzymes rather than to the presence in the tissue of other factors inhibiting or enhancing the enzymatic activities.

Summary. The activities of enzymes involved in the transmethylating pathway for the formation of lecithins (phosphatidyl choline) were determined in homogenates of the livers of rats at various stages of development. If the values are expressed per unit of protein weight, the enzyme synthesizing adenosylmethionine appears to be quite active at the end of the second week of fetal life. In contrast, values for the phospholipid methyltransferase, the enzyme catalyzing the stepwise methylation of a phospholipid (presumably phosphatidyl ethanolamine) to lecithin, remain quite low in the fetal livers, increase rapidly after birth, and reach their peak at about the eighth day of extrauterine life. This pattern is similar to that previously observed for choline phosphotransferase, the enzyme which catalyzes the final step in the cytidine-dependent pathway, except that the latter enzyme increases more markedly during the last week of fetal life, suggesting that this pathway reaches full development somewhat earlier than the transmethylating pathway.

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