BBA 55596

# PHOSPHOLIPID METABOLISM IN THE DEVELOPING LIVER

## I. THE BIOSYNTHESIS OF CHOLINE GLYCEROPHOSPHATIDES BY LIVER SLICES FROM FETAL, NEWBORN AND ADULT RATS

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(Received March 17th, 1969)

SUMMARY

1. The biosynthesis of choline glycerophosphatides was evaluated in fetal, neonatal and adult female rat liver. The two main pathways were assessed by studying the rates of incorporation of [*Me*-$^{14}$C]choline and [*Me*-$^{14}$C]methionine by liver slices.

2. Over 96% of the lipid radioactivity from [*Me*-$^{14}$C]choline was located in choline glycerophosphatides. In adult liver slices 93% of the total lipid radioactivity from [*Me*-$^{14}$C]methionine was located in choline glycerophosphatides. An appreciable amount of radioactivity from [*Me*-$^{14}$C]methionine was incorporated into fatty acids by fetal liver slices.

3. Liver slices from fetal rats incorporated the same amount of [*Me*-$^{14}$C]choline into lecithin as did adult liver slices except for a drop 2 days before birth. Liver slices from 3-day-old neonatal rats incorporated about twice as much [*Me*-$^{14}$C]choline as the adult liver slices.

4. Liver slices from fetal and young rats incorporated about 8–10 times more [*Me*-$^{14}$C]choline into phosphoryl choline than did liver slices from adults. Adult levels were reached at 10 days after birth.

5. The incorporation of radioactivity from [*Me*-$^{14}$C]methionine into lecithin by fetal liver slices was much lower than that by adult liver slices and increased to twice adult levels by 10–12 days after birth. Adult levels were reached by 25 days after birth.

6. Studies with [1,2-$^{14}C_2$, *Me*-$^{3}$H]choline indicated that the choline was incorporated intact and that little, if any, of the methyl groups of choline were used for the methylation of phosphatidyl ethanolamine.

7. The mechanism of choline incorporation into lecithin is discussed.

## INTRODUCTION

Initial studies on prenatal and postnatal rat liver showed that the total phospholipid content and ability to incorporate $^{32}P$ into phospholipids increased rapidly immediately after birth[1]. The most pronounced changes involved choline glycerophosphatides. The present investigation represents a more detailed study of the biosynthesis of choline glycerophosphatides and related metabolism of choline during prenatal and postnatal development of the rat liver. The ability of liver slices to incorporate [*Me*-$^{14}$C]choline into choline glycerophosphatides was used to measure the activity of the direct synthetic pathway[2]. The incorporation of L-[*Me*-$^{14}$C]-methionine into choline glycerophosphatides was employed to determine the activity of the pathway that involves the methylation of phosphatidyl ethanolamine[3,4]. Studies of the kinetics of incorporation into both choline glycerophosphatides and phosphoryl choline were done in order to specify possible mechanisms associated with the observed changes in the synthetic pathway.

## MATERIALS AND METHODS

Pregnant rats of specified delivery dates ($\pm$ 12 h) were purchased from the Holtzman Co., Madison. [*Me*-$^{14}$C]Choline, [1,2-$^{14}$C]choline, [*Me*-$^{3}$H]choline and L-[*Me*-$^{14}$C]methionine were obtained from New England Nuclear Corporation, Boston. Their purity was checked by paper chromatography using the solvent systems ethanol–conc. $NH_4OH$ (95:5, v/v) and phenol–water (70:30, w/v) and were found to be more than 99% pure.

Liver slices were prepared with a Stadie–Riggs microtome as previously described[1]. Slices (150–250 mg) were incubated in an atmosphere of 95% $O_2$–5% $CO_2$ in 2 ml of Krebs-bicarbonate medium (pH 7.4)[5] that contained 2 mg/ml glucose and 1 $\mu$C/ml radioactivity. The specific activity of the radioactive choline and methionine was adjusted with unlabeled material, so that a concentration in the incubation medium of 1.3 mM and 1.0 mM, respectively, was achieved.

The lipids were extracted and analyzed by methods previously reported[1]. The analysis of water-soluble products from [*Me*-$^{14}$C]choline was performed as previously reported[6] except the specific activity of phosphoryl choline was determined by extracting the phosphoryl choline area of the chromatogram with 0.1 M HCl. The 0.1 M HCl extract was passed through small columns of AG 50 $H^+$, 100–200 mesh ion exchange resin. The column was washed with water and the phosphoryl choline eluted with 2 M HCl. Radioactivity and total phosphorus was determined in the 2 M HCl eluate.

Radioactivity was determined in a liquid scintillation spectrometer. The counts were corrected to 100% counting efficiency by the channels ratio method[7]. Radioactivity in the double-label experiments was determined essentially as described by BALINT *et al.*[8].

Lipid was hydrolyzed with 6 M HCl at 110° for 8 h in sealed vials. The fatty acids were separated from the hydrolysate by 3 extractions with equal volumes of hexane.

## RESULTS

Several experiments were performed to assess the stability and validity of slice preparations for the comparison of the metabolic capabilities of fetal and adult liver. The sucrose space of slices from fetal liver and adult liver did not change over a 2-h incubation. The tissue potassium levels of fetal and adult liver slices increased during the first 30 min of incubation and thereafter remained constant for the duration of the incubation (3 h).

The incorporation of [*Me*-$^{14}$C]choline and [*Me*-$^{14}$C]methionine into choline glycerophosphatides by fetal and adult liver slices was determined with different concentrations of the precursor in the incubation medium. The incorporation of [*Me*-$^{14}$C]choline into total lipid was nearly maximal at 1.0 mM choline for both fetal and adult liver slices. A slightly higher incorporation did occur with 2.0 mM choline in the medium. The incorporation of [*Me*-$^{14}$C]choline was linear for 2 h at a choline concentration of 1.3 mM with both adult and fetal liver. The incorporation of radioactivity from [*Me*-$^{14}$C]methionine reached a maximal value at 1.0 mM. Increased concentrations gave no further increase in incorporation. The incorporation of radioactivity from [*Me*-$^{14}$C]methionine was linear for 2 h with 1.0 mM methionine in the medium.

TABLE I

CHROMATOGRAPHIC SEPARATION OF RADIOACTIVE LIPID

The fetal rats used in this experiment were 20 days gestation. Separation was done by thin-layer chromatography[1]. Each value is the average ± S.D. of 10 separate incubations and is expressed as the percentage of the total radioactivity in the lipid extract.

| *Fraction* | [*Me*-$^{14}$C]*Choline* | | [*Me*-$^{14}$C]*Methionine* | |
|---|---|---|---|---|
| | *Adult* | *Fetal* | *Adult* | *Fetal* |
| Sphingomylin | 0.51 ± 0.12 | 0.96 ± 0.33 | 0.50 ± 0.16 | 1.20 ± 0.91 |
| Lysolecithin | 1.33 ± 0.22 | 1.18 ± 0.30 | 0.88 ± 0.20 | 1.08 ± 0.38 |
| Choline glycerophosphatides | 96.40 ± 0.68 | 96.40 ± 0.55 | 93.02 ± 1.52 | 82.51 ± 3.07 |
| Inositol glycerophosphatides | 0.08 ± 0.04 | 0.12 ± 0.05 | 0.40 ± 0.07 | 0.92 ± 0.43 |
| Serine glycerophosphatides | 0.90 ± 0.54 | 0.21 ± 0.09 | 3.11 ± 0.61 | 3.97 ± 0.36 |
| Ethanolamine glycerophosphatides | 0.16 ± 0.05 | 0.16 ± 0.10 | 0.59 ± 0.29 | 2.54 ± 1.37 |
| Neutral lipids | 0.20 ± 0.11 | 0.16 ± 0.06 | 0.46 ± 0.29 | 5.40 ± 1.71 |

[*Me*-$^{14}$C]choline is incorporated almost exclusively into choline glycerophosphatides (Table I). The radioactivity from [*Me*-$^{14}$C]methionine is incorporated predominantly into choline glycerophosphatides with both adult and fetal liver slices. However, there is an appreciable amount of radioactivity located in the serine glycerophosphatide area of the chromatogram. This is probably in monomethyl and dimethyl ethanolamine glycerophosphatides since these compounds migrate in this region. A lower percentage of the total radioactivity is found in choline glycerophosphatides in fetal liver than in adult liver, and a higher percentage is found in ethanolamine glycerophosphatides and in neutral lipids. Although the incorporation of radioactivity into total lipid is decidedly lower in fetal than in adult liver, the incorporation into the neutral lipids is higher in fetal than adult when expressed on a tissue wet weight basis (9200 disint./min per g for adults and 21600 disint./min per g for 21-day fetal). The incorporation of radioactivity into ethanolamine glycerophosphatide from fetal liver does not differ from that from adult liver.

TABLE II

ACID HYDROLYSIS OF TOTAL RADIOACTIVE LIPID

| | *Hexane* | | *Aqueous* | |
|---|---|---|---|---|
| | % | *disint./min per mg tissue* | % | *disint./min per g tissue* |
| [*Me*-$^{14}$C]Choline | | | | |
| Adult | 0.2 | 2 | 99.8 | 666 |
| Fetal (18)* | 0.8 | 7 | 99.2 | 835 |
| [*Me*-$^{14}$C]Methionine | | | | |
| Adult | 0.7 ± 0.1** | 13 ± 2 | 99.3 ± 0.1 | 1762 ± 36 |
| Fetal (20)* | 7.8 ± 0.2 | 24 ± 2 | 92.2 ± 0.2 | 282 ± 31 |

* Days gestation.
** Standard deviation for 3 separate incubations.

There was practically no incorporation of radioactivity from [*Me*-$^{14}$C]choline into fatty acids (Table II). Adult liver slices incorporated some radioactivity from [*Me*-$^{14}$C]methionine into the hexane-soluble portion of the acid hydrolysate of total lipid. Noticeably higher amounts of radioactivity from [*Me*-$^{14}$C]methionine was found in the hexane fraction from the hydrolyzed lipid from fetal liver slices. The hexane-soluble material from both adult and fetal lipid was fractionated by thin-layer chromatography on silica gel H with the solvent system light petroleum–diethyl ether–acetic acid (90:10:1, v/v/v)[9]. Free fatty acids accounted for 51–53% of the hexane-soluble radioactivity. 37–40% of the radioactivity migrated near the solvent front. There was no radioactivity or detectable lipid in the region where triglycerides and phospholipids migrate, and the distribution of radioactivity was similar in both adult and fetal lipid. Paper chromatography of the aqueous portion from the acid hydrolysis indicated that all of the radioactivity was associated with

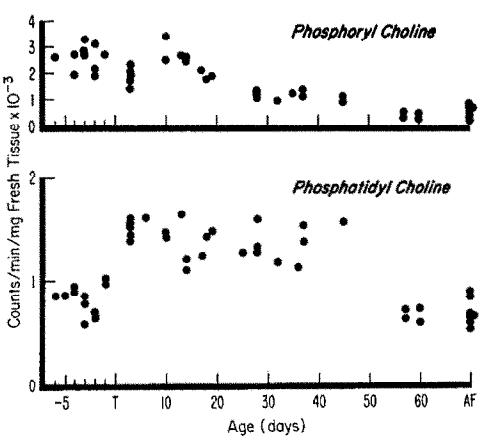


Fig. 1. Incorporation of [*Me*-$^{14}$C]choline into phosphoryl choline and choline glycerophosphatides. The points represent the amount of radioactivity incorporated into isolated choline glycerophosphatides and phosphoryl choline during a 60-min incubation of liver slices. Each is the average of two separate incubations from either the combined livers from a litter of fetal rats or from the liver of an infant or adult rat. Infant rats were randomly selected from the litter until they were weaned (20 days) after which only female rats were used. All adult rats were female. T = term; AF = adult female.

choline in both adult and fetal lipid when either [*Me*-$^{14}$C]choline or [*Me*-$^{14}$C]methionine was used as a precursor.

Liver slices from fetal rats at 17, 18 and 19 days gestation (5, 4 and 3 days before birth) incorporate [*Me*-$^{14}$C]choline to the same extent as adult liver slices (Fig. 1). The ability to incorporate [*Me*-$^{14}$C]choline drops at 20–21 days gestation and then increases to levels slightly higher than adult at 22 days gestation. Liver slices from 3-day-old rats incorporate [*Me*-$^{14}$C]choline at a rate twice that of the adult slices. This higher level of incorporation continues for the next 25–30 days before returning to adult levels by 57 days. Liver from fetal and young rats incorporated about 8–10 times more [*Me*-$^{14}$C]choline into phosphoryl choline than did liver slices from adult females. The amount of radioactivity in the phosphoryl choline begins to decline toward adult levels at 10 days before birth and reaches adult values by 28 days.

A series of experiments were performed with double-labeled choline (a mixture of [1,2-$^{14}C_2$]choline and [*Me*-$^{3}$H]choline). The $^{3}H/^{14}C$ ratio was determined for the original choline added to the medium, for phosphoryl choline isolated from the slice and for choline isolated from the hydrolyzed lipid. The incorporation by liver slices from fetal rats 20 and 21 days gestation and from 2-day-old rats and nonpregnant female rats was determined. In all experiments the $^{3}H/^{14}C$ ratios in phosphoryl choline and lipid choline were the same as those of the choline added to the medium (14.4, 14.1 and 14.5, respectively).

The amount of incorporation of [*Me*-$^{14}$C]choline into phosphoryl choline and choline glycerophosphatides was determined after various lengths of incubation. The results of these experiments with fetal liver (20 and 22 days gestation), neonatal

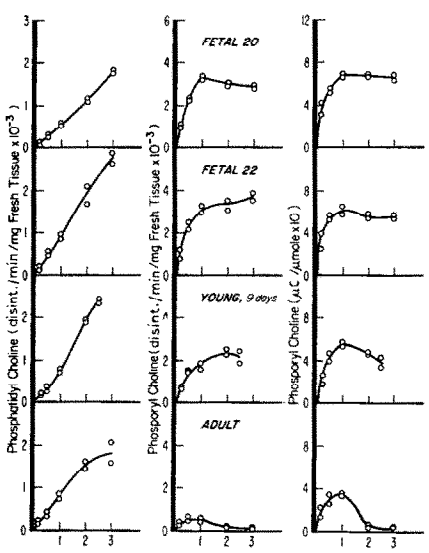


Fig. 2. The incorporation of [*Me*-$^{14}$C] choline into phosphoryl choline and choline glycerophosphatides after different lengths of incubation. Each point is the value from a single incubation. All fetal experiments used the combined livers from 2 litters of rats. The experiment with 9-day-old rats used the combined livers from three randomly selected rats from the same litter. The adult experiments used the liver from a single female rat.

liver (9 days old) and adult female liver are shown in Fig. 2. The incorporation into choline glycerophosphatides is linear for 3 h with fetal liver and neonatal liver, but the incorporation by adult liver drops slightly after 2 h. The total amount of radioactivity incorporated into phosphoryl choline by fetal liver reaches a maximum by 60 min and remains practically constant to 3 h. The specific activity of phosphoryl choline also reaches a maximum at 60 min. The maximum specific activity with 20- and 22-day fetal liver (0.6 to 0.68 $\mu$C/$\mu$mole) approaches the specific activity of the choline in the incubation medium (0.77 $\mu$C/$\mu$mole). Adult liver slices incorporate much less radioactivity into phosphoryl choline than fetal and neonatal liver slices. The incorporation by adult slices of [*Me*-$^{14}$C]choline into phosphoryl choline reaches a maximum at 1 h but then drops, so that by 2 h very little radioactivity can be detected in phosphoryl choline. The specific activity rises to a maximum value by 1 h and also drops to low levels by 2 h. The experiment with adult liver slices was repeated several times with similar results.

TABLE III

CONCENTRATION OF PHOSPHORYL CHOLINE IN FETAL AND ADULT LIVER

The concentrations are expressed as $\mu$moles/g tissue and were calculated from the total radioactivity in the phosphoryl choline and the specific activity of the phosphoryl choline from Fig. 2.

| *Age* | *Incubation time (min)* | | | | |
|---|---|---|---|---|---|
| | *10* | *30* | 60 | *120* | *180* |
| Fetal 20 | 1.30 | 1.95 | 2.20 | 2.00 | 2.00 |
| Fetal 22 | 1.42 | 2.02 | 2.20 | 2.68 | 3.00 |
| Adult female | 1.66 | 1.66 | 1.47 | 1.17 | 1.25 |

The concentration of phosphoryl choline in fetal and adult liver after various periods of incubation is shown in Table III. The concentration in 20-day fetal liver increases up to 60 min of incubation and remains practically constant for the remaining 2 h of incubation. The concentration in 22-day fetal liver continues to increase. The concentration in adult slices is higher than in fetal slices after 10-min incubations. However, the concentration does not increase with increased length of incubation but instead decreases after 30 min.

The incorporation of radioactivity from [*Me*-$^{14}$C]methionine is very low in slices of liver from fetal rats at 19 days gestation (Fig. 3). There is a slight increase with increased fetal age. The ability to utilize the methyl groups from methionine increases rapidly in early postnatal rats and reaches a maximum at 10–12 days after

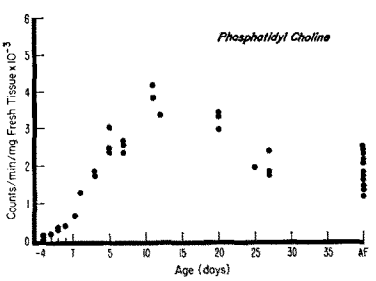


Fig. 3. Incorporation of [*Me*-$^{14}$C]methionine into choline glycerophosphatides. For details see the legend for Fig. 1.

birth. The maximum ability is about twice the value for the adult female. Adult levels are attained by 25 days after birth.

## DISCUSSION

It is usually assumed that [*Me*-$^{14}$C]choline is incorporated into choline glycerophosphatides only by the direct CDP-choline pathway[2]. There are two other known ways in which radioactivity from [*Me*-$^{14}$C]choline could be incorporated into choline glycerophosphates. The methyl group could be relocated to methionine during the oxidation of choline[10] and then used in the methylation of ethanolamine glycerophosphatides to form choline glycerophosphatides[3] or [*Me*-$^{14}$C]choline could be incorporated into choline glycerophosphatides by an exchange reaction similar to that observed to occur in a system *in vitro*[11]. The results from the double-label experiments indicated that there is little, if any, utilization of methyl groups from choline for the methylation of ethanolamine glycerophosphatides. The direct exchange reaction cannot be ruled out. The results in Fig. 2 with fetal liver slices are as expected for the incorporation of [*Me*-$^{14}$C]choline into choline glycerophosphatides through phosphoryl choline. The total radioactivity in phosphoryl choline increases to a maximal level and remains at that level and the specific activity of phosphoryl choline approaches the specific activity of the added choline. However, the results with adult liver slices are not as easy to interpret. The total radioactivity in phosphoryl choline does not remain at a maximal level nor does the specific activity of phosphoryl choline approach the specific activity of the added choline. In fact, the specific activity of the phosphoryl choline drops after 2 h incubation to very low levels. However, the incorporation of radioactivity into choline glycerophosphatides does not drop between 1 and 2 h of incubation, but continues to increase at about the same rate as occurred during the first hour of incubation. Obviously, there are aspects in the metabolism of choline and choline glycerophosphatides in the adult liver that do not occur in fetal liver and apparently are only beginning to influence the results in 9-day-old rat liver. Although no definite conclusion concerning these differences can be drawn from the present data, some possibilities can be considered It is important to note that although the concentration of phosphoryl choline in adult liver does decline during the incubation (Table III), this drop is not sufficient to explain the drop in radioactivity nor can it account for the drop in the specific activity of phosphoryl choline. The increase in the radioactivity in choline glycerophosphatides from 1 h incubation to 2 h incubation is more than can be accounted for by the drop in radioactivity in phosphoryl choline. Therefore, there appears to be a sufficient supply of choline in the tissue to provide phosphoryl choline and the specific activity of this choline must be dropping rapidly during the incubation. This drop in specific activity cannot be entirely caused by dilution from the hydrolysis of choline glycerophosphatides, since it would require 17.4 $\mu$moles of choline to dilute the specific activity of the added choline from 0.77 $\mu$C/$\mu$mole to 0.1 $\mu$C/$\mu$mole, and there is only 7.4 $\mu$moles of choline glycerophosphatides in 200 mg of fresh rat liver[12,13]. If an average specific activity of the choline is assumed to be 0.3 $\mu$C/$\mu$mole, only 0.6 $\mu$mole of choline is utilized for lipid synthesis during 2 h of incubation. Therefore, the pronounced drop in specific activity of the phosphoryl choline must be caused by a rapid metabolism of [*Me*-$^{14}$C]choline by another pathway so that after 1 h of

incubation the amount of radioactive choline is such that the choline produced by the liver slice is sufficient to dilute the specific activity to low levels. Indeed, when the water-soluble material from the combined slice and incubation medium was analyzed by paper chromatography, very little radioactivity was found in free choline after 1 h incubation, and by 2 h of incubation no radioactive choline could be found. Instead, about 90% of the total water-soluble radioactivity was associated with a metabolite, which has not yet been positively identified (unpublished observations). Why the radioactivity in choline glycerophosphatides continues to increase despite the disappearance of radioactive choline is unexplained. One possible explanation would be to assume that there are at least two separate pools of phosphoryl choline in the cell. One pool is small compared with the total phosphoryl choline, and exogenously supplied choline is utilized only for the production of this phosphoryl choline.

The incorporation of [*Me*-$^{14}$C]methionine into choline glycerophosphatides is quite low in fetal liver and implies that the synthesis of choline glycerophosphatides by the methylation of ethanolamine glycerophosphatides is not very active until 1–3 days after birth. Since this pathway is believed to be responsible for the synthesis of choline[3], the fetal rat apparently has little ability to synthesize choline and therefore must rely on the mother for its choline supply.

The utilization of the methyl group of methionine for the synthesis of fatty acids has not previously been reported. The oxidation of [*Me*-$^{14}$C]methionine to $^{14}CO_2$ by the rat has been known for many years[14], however, the mechanisms involved are not fully understood. Presumably, the methyl groups from methionine enter the "one-carbon pool" and are combined through 5,10-methylene tetrahydrofolic acid with glycine to form serine. Serine could be converted to pyruvic acid by serine dehydrase. The pyruvic acid would be converted to acetyl-CoA which could then either be oxidized by the citric acid cycle or be used for the synthesis of fatty acids. All of the above reactions are known to occur in rat liver[15]. VILLEE AND HAGERMAN[16] have shown that [2-$^{14}$C]pyruvate is incorporated into lipid at a considerably higher rate by liver slices from fetal rats than by adult liver slices. This could account for the higher incorporation of radioactivity from [*Me*-$^{14}$C]methionine into fatty acids that was observed in the present fetal liver slices.

BALDWIN AND CORNATZER[17] have found the activities of CDP-choline–diglyceride transferase and phosphatidyl methyl transferase to be low in fetal rabbit liver. The phosphatidyl methyl transferase activity, used to measure the methylation pathway, reached adult levels shortly after birth. The CDP-choline–diglyceride tranferase activity reached adult levels at 9 days after birth. Thus, the developmental pattern of these two enzymes in rabbit liver closely parallels the pattern of change observed here in the two pathways in rat liver.

The results from the double-label experiments indicate that there is little if any utilization of the methyl groups from choline for the subsequent methylation of ethanolamine glycerophosphatides, and thus the changing activity of the methylation pathway does not contribute to the observed pattern of incorporation of radioactivity from [*Me*-$^{14}$C]choline.

The increased activity for the synthesis of choline glycerophosphatides during the latter 2 days of gestation and early newborn period coincides with the observed increase in the amount of intracellular membrane in the liver[18]. Thus, the liver cell

would be able to synthesize choline glycerophosphatides at an increased rate when the requirement of phospholipids for membrane formation and turnover was increasing. Phosphoryl-choline–CTP transferase and CDP-choline–glyceride transferase are located in the endoplasmic reticulum[18,19], and the amount of these could increase with the increased extent of intracellular membranes. However, DALLNER *et al.*[20] have shown that the appearance of other constitutive enzymes of microsomal membranes does not necessarily coincide with the pattern of increase in total membrane but may appear at different times and increase at different rates.

The liver, in addition to synthesizing phospholipids for membrane formation, also supplies phospholipids to the serum and bile[21–24]. The developmental pattern of synthesis of choline glycerophosphatides may also be related to these functional requirements.

The interpretation of biochemical data from fetal rat liver is complicated by the high hemopoietic activity in fetal rat liver. The amount of hemopoietic tissue in fetal liver decreases uniformly from about 60% at 17 days gestation to adult levels of less than 10% by 6 days after birth[25]. Although this is no doubt a factor in the pattern observed here for phospholipid synthesis, the changes in the ability of liver slices to synthesize choline glycerophosphatides do not coincide with this pattern of change in cellular composition of the liver.

ACKNOWLEDGMENTS

The author wishes to thank Mr. Robert Cleveland and Mr. Jay Hall for their excellent technical assistance. This work was aided by Grant HD-02871 from the National Institutes of Health, U.S. Public Health Service.

REFERENCES

1 P. A. WEINHOLD AND C. A. VILLEE, *Biochim. Biophys. Acta*, 106 (1965) 540.
2 E. P. KENNEDY, *Federation Proc.*, 20 (1961) 934.
3 J. BREMER, P. H. FIGARD AND D. M. GREENBERG, *Biochim. Biophys. Acta*, 43 (1960) 477.
4 J. D. WILSON, K. D. GIBSON AND S. UDENFRIEND, *J. Biol. Chem.*, 235 (1960) 3213.
5 H. A. KREBS AND K. HENSELEIT, *J. Physiol. Chem.*, 210 (1932) 33.
6 P. A. WEINHOLD, *J. Lipid Res.*, 9 (1968) 262.
7 E. T. BUSH, *Anal. Chem.*, 35 (1963) 1024.
8 J. A. BALINT, D. A. BEELER, D. H. TREBEL AND H. L. SPITZER, *L. Lipid Res.*, 8 (1967) 486.
9 D. C. MOLINS AND H. K. MANGOLD, *J. Am. Oil Chemists' Soc.*, 37 (1960) 576.
10 D. M. GREENBERG, in D. M. GREENBERG, *Metabolic Pathways*, Vol. 2, Academic Press, New York, 1961, p. 184.
11 R. R. DILS AND G. HÜBSCHER, *Biochim. Biophys. Acta*, 46 (1961) 505.
12 M. M. RAPPORT AND B. LERNER, *Biochim. Biophys. Acta*, 33 (1959) 319.
13 G. V. MARINETTI, J. ERBLAND AND E. STOTZ, *Biochim. Biophys. Acta*, 30 (1958) 642.
14 C. G. MCKENZIE, J. P. CHANDLER, E. B. KELLER, J. R. RACHELE, N. CROSS, D. B. MELVILLE AND V. DUVIGNEAUD, *J. Biol. Chem.*, 169 (1947) 757.
15 E. S. WEST, W. R. TODD, H. S. MASON AND J. T. VAN BRUGGEN, *Textbook of Biochemistry*, MacMillan Company, New York, 1967.
16 C. A. VILLEE AND D. D. HAGERMAN, *Am. J. Physiol.*, 194 (1958) 457.
17 J. BALDWIN AND W. E. CORNATZER, *Lipids*, 3 (1968) 361.
18 G. DALLNER, P. SIEKEVITZ AND G. E. PALADE, *J. Cell. Biol.*, 30 (1966) 73.
19 G. F. WILGRAM AND E. P. KENNEDY, *J. Biol. Chem.*, 238 (1963) 2615.
20 G. DALLNER, P. SIEKEVITZ AND G. D. PALADE, *J. Cell. Biol.*, 30 (1966) 97.
21 M. D. FISCHLER, C. ENTENMAN, M. L. MONTGOMERY AND I. L. CHAIKOFF, *J. Biol. Chem.*, 150 (1943) 47.
22 G. POPJAK AND H. MUIR, *Biochem. J.*, 46 (1950) 103.
23 B. BORGSTRÖM AND T. OLIVECRONA, *J. Lipid Res.*, 2 (1961) 263.
24 D. B. ZILVERSMIT AND E. VAN HANDEL, *Arch. Biochem. Biophys.*, 73 (1958) 224.
25 J. T. OLIVER, W. F. C. BLUMER AND J. J. WITHAM, *Comp. Biochem. Physiol.*, 10 (1963) 33.