Biosynthesis of retinal phospholipids: incorporation of radioactivity from labeled phosphorylcholine and cytidine diphosphate choline

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ABSTRACT Phosphorylcholine-1,2-^{14}C and choline-1,2-^{14}C-labeled cytidine diphosphate choline are incorporated into lecithin by whole homogenates and particulate fractions of rat retina with optimal incorporation of label by the microsomal fraction. The soluble fraction contains a factor(s) which stimulates incorporation of label with release of inorganic phosphate. Mg^{++} is required for optimal incorporation of intermediates into lecithin in the presence of added diglycerides; without added diglycerides, incorporation of phosphorylcholine or cytidine diphosphate choline was moderately stimulated by preincubating the system in the absence of Mg^{++} with added phosphatidic acid and by adding this mixture to fresh enzyme and the complete incubation mixture (including Mg^{++}). The results show that the retina is capable of *de novo* synthesis of phosphatides and suggest that the rod outer segments depend on the pigment epithelium and(or) the inner rod segments for a source of phospholipids. Coenzyme A and ATP added to whole homogenate of retina did not significantly increase the incorporation of CDP-choline-1,2-^{14}C into lecithin but slightly increased the radioactivity found in lysolecithin and sphingomyelin. Rats with hereditary retinitis pigmentosa have an abnormally high lipid phosphorus content of the retina, but they do not incorporate labeled CDP-choline into lecithin of retina at a higher rate than do normal animals.

SUPPLEMENTARY KEY WORDS cytidyl transferase · glyceride transferase · retinitis pigmentosa

THE LECITHIN content of the retina of several species has been known for almost a century (1, 2), and although recent studies (3–9) have emphasized the high total lipid content of retinae and their rod outer segments, few attempts have been made to resolve and identify individual retinal phospholipids (4) or to trace the origin of these compounds. This investigation was initiated to test retinal cellular fractions from normal and dystrophic rat retinae for cytidyl transferase and glyceride transferase activity (10–12) in an effort to trace the biogenesis of retinal phospholipids. We report here on the conditions of incorporation of labeled intermediate compounds into phospholipids by enzyme systems present in retinal tissue fractions.

METHODS

Retinae were obtained from Sprague-Dawley albino rats weighing 150–200 g each and from the RCS strain of piebald, agouti rat suffering an hereditary retinal degeneration described by Bourne, Campbell, and Tansley (13), by Dowling and Sidman (14), and by Sidman, Pearlstein, and Waymouth (15). Globe excisions were performed on animals under sodium pentothal anesthesia, and the animals were killed with a lethal dose of tubocurarine chloride. The retinas were immediately removed and homogenized in Tris–HCl buffer or 0.25 M sucrose at 0°C in a Potter–Elvehjem type homogenizer. Particulate fractions were obtained according to methods and suggestions by Schneider (16), by Abood and Gerard (17), and by Abood, Gerard, Banks, and Tschirgi (18), using

Abbreviations: CDP-choline, cytidine diphosphate choline; PC, phosphorylcholine; PE, phosphatidylethanolamine; CTP, cytidine triphosphate; PA, phosphatidic acid; ATP, adenosine triphosphate; CoA, coenzyme A; RCS, Royal College of Surgeons; TLC, thin-layer chromatography.

high-speed, refrigerated centrifuges (model HR-1; International Equipment Co., Needham Heights, Mass., and Beckman Spinco model L with a SW 50.1 rotor; Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Mitochondria were obtained at 15,000 *g*, and microsomes at 108,000 *g*. The "fluffy" layer was not used. Rod outer segment fractions were isolated using the method of Poincelot and Zuli (19). Cross-contamination was followed by assaying fractions for cytochrome c oxidase activity (20, 21) and glucose-6-phosphatase activity (22).

Aliquots of whole homogenate were added to a basic medium and incubated for 1 hr at 37.5°C under ambient conditions in a Dubnoff metabolic shaker. Media containing particulate fractions were incubated in an O_2 atmosphere. The basic incubating medium consisted of the following in a total volume of 2.0 ml: $MgCl_2$, 20 μmoles; Tris buffer, pH 7.36, 100 μmoles; radioactive precursor at a concentration to give approximately 10^6 cpm/μmole; and tissue homogenate or cell fraction equivalent to 25–100 mg (wet weight) of tissue. The usual procedure was to add the equivalent of 50 mg of fresh tissue to each beaker. Other constituents were added as indicated in the Tables or Figures.

The lipid extraction procedure was that of Vorbeck and Marinetti (23); all extractions were carried out under a nitrogen atmosphere. Nonlipid contaminants were removed from the final lipid extract by passing the extract over a Sephadex column, as outlined by Wells and Dittmer (24). The importance of this step in obtaining reliable final results cannot be overemphasized.

Tissue lipid extract and reference compounds were applied to Silica Gel G-coated TLC plates obtained from Analtech, Inc., of Wilmington, Del., and silica gel-loaded paper (25) from H. Reeve Angel & Co., Inc., Clifton, N.J. TLC plates were developed in chloroform–methanol–water 60:25:4; silica gel papers were developed in diisobutyl ketone–acetic acid–water 40:25:5. Two-dimensional chromatograms were developed in direction I with chloroform–methanol–5 M ammonia 90:45:11, and in direction II with chloroform–methanol–acetic acid–water 90:40:12:2. Individual spots from chromatograms were analyzed quantitatively according to the methods of Rosenthal and Han (26) and Parker and Peterson (27). The lipid spots on the chromatograms were visualized with the following reagents (singly or in combination): vapors from iodine dissolved in methanol, 1% phosphomolybdic acid and 1% stannous chloride dip (paper), 0.1% phosphomolybdic acid and 0.1% stannous chloride spray (TLC plates), rhodamine 6G (viewing with UV light), 2,4-dinitrophenylhydrazine, and 0.25% ninhydrin in acetone–lutidine 9:1.

Radioactivity on paper and thin-layer chromatograms was measured using a radiochromatogram scanner equipped with a recorder and disc integrator (Packard model 7201). Aliquots of extract or material obtained from identified spots on the chromatograms were plated at "infinite" thinness on aluminum planchets, dried, and counted in a gas-flow beta counter (model D-47; Nuclear-Chicago Corporation, Des Plaines, Ill.).

Identification of individual phospholipids was based on the following criteria: (*a*) R_F value of resolved material compared with R_F value of purified, synthetic reference compounds; (*b*) hydrolysis products of material scraped or cut from chromatograms (mild hydrolysis was carried out according to Dawson [28]); (*c*) color reactions of individual spots of material with the reagents listed above; (*d*) the presence or absence of a "quenching" effect of compounds exposed to UV light.

The lipid phosphorus content of samples was determined by the method of Bartlett (29). Samples were read at 700 nm using a Beckman model DB spectrophotometer. *p*-(Methylamino) phenol sulfate (Elon reagent) used as reducing agent with the phosphoric–molybdate complex gave a highly stable color complex, but in our hands this reagent did not provide the sensitivity given by 1-amino-2-naphthol-4-sulfonic acid (ANSA reagent). Standard phosphate and phospholipid (recovery) determinations were made with each set of "unknown" extracts which were analyzed, and results were read from a standard phosphate curve prepared with each analysis. Recovery of known quantities of phospholipid was 83–98%. Final results were calculated to adjust for any loss of phosphorus during analysis.

Permanent records of developed chromatograms were made by a radioautographic technique using high-speed X-ray film with an exposure time of 3 days, and blueprint records were obtained by modifying the method of Zeitman (30) to employ dilute ammonia dips in place of concentrated ammonia vapor.

Phosphorylcholine-1,2-^{14}C was obtained from International Chemical & Nuclear Corporation (ICN), Burbank, Calif., and all other radioactive compounds were from Tracerlab Inc., of Richmond, Calif. Nonlabeled reference compounds were obtained from Pierce Chemical Co. of Rockford, Ill. Soybean lecithin and lecithinase from *Clostridium perfringens* were products of Nutritional Biochemicals Corporation of Cleveland, Ohio. The purity of reference compounds was checked by thin-layer chromatography.

Boiled enzyme preparation, that is, homogenate, mitochondria, or microsomes, added to the incubating medium served as the control in each experiment and insured a constant protein content in all incubation mixtures. Unless otherwise stated in the text, the radioactivity detected in phospholipids from the control sample did not exceed background radioactivity.

RESULTS

Table 1 shows that the radioactivity of phosphorylcholine-^{14}C and CDP-choline-^{14}C was incorporated into the lecithin of homogenates and particulate fractions of rat retina. For optimal incorporation of label, an oxygen atmosphere was needed to support mitochondrial reactions that ultimately lead to formation of labeled lecithin by microsomes. There was a requirement for CTP for optimal incorporation of PC into lecithin, but addition of CoA did not increase the radioactivity found in lecithin. With CDP-choline as the labeled intermediate, the homogenate was the most active in forming labeled lecithin. Addition of ATP to homogenate and mitochondrial fractions resulted in only a slight increase in the radioactivity of lecithin. CoA slightly depressed the incorporation of label in the mitochondrial preparations but increased the activity from CDP-choline by as much as 30% in homogenates. In whole homogenate preparations or on addition of soluble fraction to a mitochondrial–microsomal preparation, there was a significant release of inorganic phosphate during incubation. We have tentatively interpreted this as being due to an hydrolysis of PA formed by microsomes, thus providing diglycerides to participate as a PC acceptor.

The derived radioactive product, synthetic L-α-lecithin, and purified egg lecithin were compared chromatographically using techniques and materials described in Methods. The R_F values of the three compounds did not vary more than 0.01. Mild hydrolysis (28) of the radioactive material yielded labeled glycerophosphorylcholine (GPC) in the alkali-labile fraction, nonlabeled GPC in the acid-labile fraction, and no detectable lipid phosphorus in the alkali-, acid-stable fraction. The isolated labeled and nonlabeled GPC represented 84% and <3%, respectively, of the total lipid phosphorus of the parent compound. Lysolecithin-^{14}C and a small amount of lecithin-^{14}C were the only compounds isolated after treating the derived product with *Crotalus adamanteous* venom (31).

TABLE 1 INCORPORATION OF RADIOACTIVITY FROM PHOSPHORYLCHOLINE-^{14}C AND CDP-CHOLINE-^{14}C INTO THE LECITHIN OF RAT RETINAL PREPARATIONS

Additions	^{14}C in Lecithin
	cpm/μg lipid phosphorus
Homogenate	
PC	2
PC plus CTP (3.5×10^{-4} M)	104
CDP-choline	1733
Mitochondria	
CDP-choline	570
CDP-choline plus ATP (10^{-5} M)	582
CDP-choline plus CoA (5×10^{-5} M)	563
Microsomes	
CDP-choline	1035
Soluble fraction	
CDP-choline	40
Mitochondria plus soluble fraction	
CDP-choline	624
Microsomes plus soluble fraction	
CDP-choline	1226

The incubation mixture contained the basic medium in a final volume of 2.0 ml. Each figure represents the average obtained from a minimum of six experiments.

Evidence that the reactions are enzymatically mediated and that CDP-choline is indeed an obligatory intermediate in the synthesis of lecithin in the retina was based on the following observations: (*a*) boiled retinal preparations did not incorporate the intermediate into lecithin, (*b*) diluting the labeled intermediates with an equal quantity of their unlabeled counterparts reduced the radioactivity detected in lecithin by approximately 50% and diluting 0.2 μmole of a labeled substrate with 1 μmole of its unlabeled counterpart could completely depress the uptake of label into lecithin, and (*c*) substrates that possibly could be hydrolysis products or precursors of CDP-choline and could participate in a nonspecific reaction were incorporated at a level much below that of CDP-choline. The lecithin that was isolated after incubating choline-^{14}C with active retinal enzyme preparations showed an activity of <1 cpm/μg of lipid phosphorus. The results obtained when PC-^{14}C was incubated in the absence of CTP are given in Table 1.

Weiss and Kennedy (32) reported that the addition of a mixture of D-α,β-diglycerides to a liver enzyme system greatly stimulated the incorporation of labeled CMP-phosphorylcholine into lecithin, with a net synthesis of lecithin. If α,β-diglycerides serve as PC acceptors in the retina, addition of appropriate acceptors to an in vitro system should stimulate the incorporation of PC from CDP-choline into lecithin. 1,2-Diolein (1 μmole), emulsified in 0.025% Tween 20 and added to retinal homogenate, increased the radioactivity of lecithin by 12% over a medium containing no glycerides. Tween 20, added as an emulsifier, does not inhibit the reaction at the final concentration used in these experiments, but as the concentration reaches 0.05–0.10%, the compound inhibits the uptake of label. The major fatty acids of yeast and soybean lecithin are palmitoleic and linoleic acids (33). We have subjected a commercial preparation of soybean lecithin to lecithinase from *C. perfringens* toxin (34), and after removal of protein from the mixture, the partially purified products were used as a source of diglycerides. As compared with diolein, the soybean diglycerides increased the incorporation of label into lecithin from 5- to 20-fold, with optimum effect at a concentration of approximately 15 mM.

D-α,β-Dipalmitin and D-α,β-distearin always stimulated the incorporation of ^{14}C from intermediates above the control level, but their effect was unexplainably

variable from experiment to experiment. L-α,β-Diolein, in contrast to D-α,β-diolein, gave results similar to those experienced with dipalmitin and distearin. Additional experimentation is needed to test the effectiveness of the diglycerides studied as substrates for diglyceride kinase, thereby ruling out the possible effects of solubility differences among the diglycerides included in our study.

Fig. 1 shows a composite blueprint of the thin-layer chromatographic separation of components of lipid extracts of the diolein media supplemented with ATP and CoA. Radioautograms of the TLC plates from which the blueprint in Plate II was made show evidence of small quantities of radioactivity in lysolecithin and sphingomyelin. One pathway for the biosynthesis of sphingomyelin proceeds via the transfer of phosphorylcholine of CDP-choline to ceramide (35). We do not know the origin of retinal sphingomyelin, but it is most probably associated with the deep, neural layers of the tissue.

Smith, Weiss, and Kennedy (36) reported that liver and brain preparations contained L-α-phosphatidic phosphohydrolase that catalyzed the following reaction:

$$\text{L-}\alpha\text{-phosphatidic acid} \rightarrow \text{D-}\alpha,\beta\text{-diglyceride} + P_i$$

Rossiter, McMurray, and Strickland (37) found that the brain phosphohydrolase is inhibited by magnesium ions. Retinal homogenate was incubated with and without synthetic phosphatidic acid (1 μmole) in the absence of an exogenous supply of Mg^{++}, and after 30 min this preparation was added to the basic medium with fresh homogenate and incubated for another 30 min. The phosphatidic acid increased the incorporation of label into lecithin by

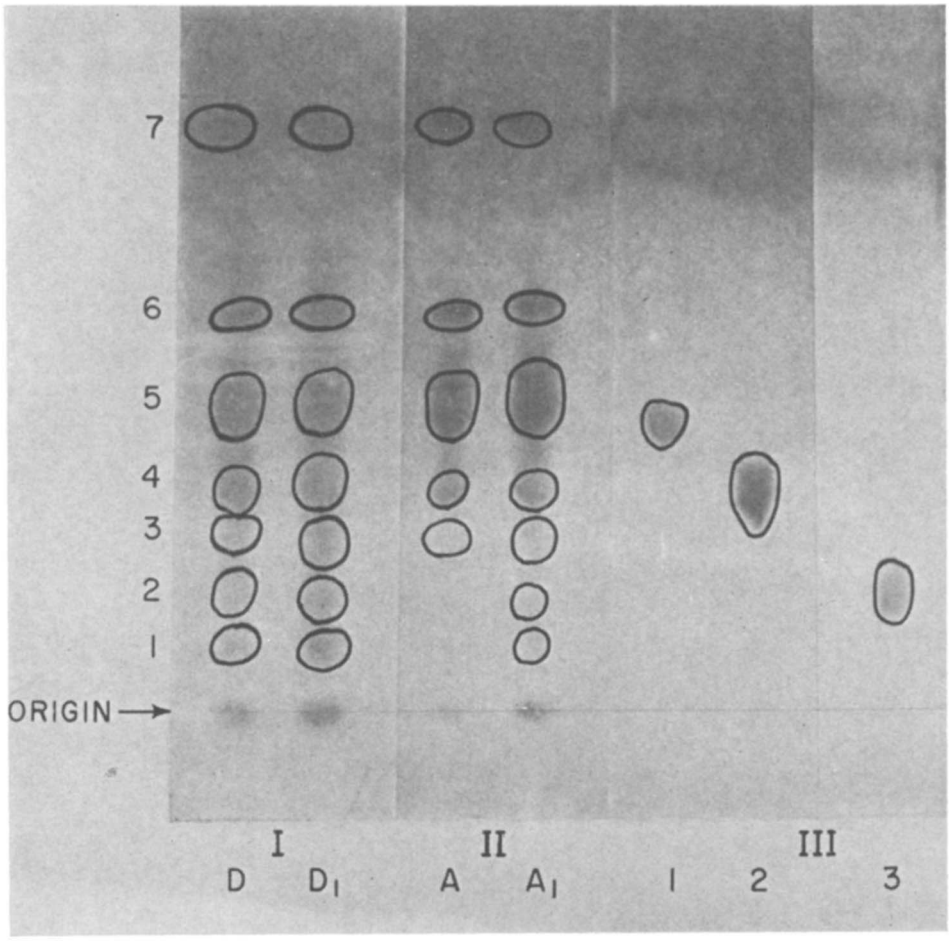

FIG. 1. Blueprint of composite one-dimensional chromatograms of rat retinal lipids from whole homogenate and reference compounds exposed to the vapor of iodine crystals dissolved in methanol. The solvent system was chloroform–methanol–water 65:25:4. Dietzgen SF Diazo blueprint paper was exposed for 4 min on Plate I and for 6 min on Plates II and III. Plate I: D, incubation in basic medium; D_1, same fraction incubated with diolein as described in text. Plate II: A, incubation in basic medium; A_1, incubation with ATP and CoA. Plate III: column 1, lecithin; column 2, sphingomyelin; column 3, lysolecithin. Identification of reference spots and spots on Plates I and II is as follows: 1, unidentified; 2, lysolecithin; 3, inositol phosphatide; 4, sphingomyelin; 5, lecithin plus plasmologens; 6, phosphatidylethanolamine 7, neutral lipids and an unidentified phospholipid. Material between spots 6 and 7 is a methyl derivative of phosphatidylethanolamine. Material between spots 5 and 6 is lyso-PE and PS.

only 6.5% over that of the basic medium alone; without PA, the increase was 4%.

The Mg^{++} requirement for the enzymes participating in the incorporation of CDP-choline into the lecithin of retinal preparations is shown in Table 2. The optimal Mg^{++} concentration for the enzymes studied is very similar to the requirement for the PC–cytidyl transferase and PC–glyceride transferase of liver (38). The inhibition by Mg^{++} at the higher concentrations was overcome by the addition of EDTA to remove calcium ions. Mn^{++}, Li^{++}, Ca^{++}, and Ba^{++} were tested as replacements for Mg^{++} in the incorporation of intermediates into lecithin. These ions however do not support the incorporation thus ruling out the possibility that Mg^{++} is serving as a non-specific ion. In the absence of the Mg^{++}, 0.02 M Ca^{++} promotes an increase of radioactivity in the lysolecithin fraction with a concomitant decrease in the concentration of labeled lecithin.

CDP-choline was incorporated into lecithin over a wide pH range; the optimal pH was approximately 7.4. Tris–HCl buffer was used to obtain values above pH 7.1, and HCl–sodium citrate buffer was used for values below pH 7.1. Phosphate buffer systems were not used in view of a much earlier report (39) that in phosphate buffer, retinal tissue does not oxidize certain substrates of the citric acid cycle. A bicarbonate buffer moderately inhibited the incorporation of intermediates.

Our results are consistent with the premise that both PC and CDP-choline are incorporated into the lecithin of mitochondria and microsomes of retina, with the rate of incorporation higher in the microsomal fraction. Wilgram and Kennedy (38) reported that CDP-choline:1, 2-diglyceride choline-phosphotransferase is located only in the microsomes. Other investigators have reported that PC is incorporated into lecithin by mitochondrial preparations (40), and that isolated mitochondria incorporate fatty acids into lecithin (41). Akiyama and Sakagami (42) recently reported that lecithin and cephalin in mitochondria could be derived from microsomes through exchange and transfer reactions, and Wirtz and Zilversmit (43) showed that phospholipids could be exchanged between liver mitochondria and microsomes in vitro.

We have not been able to obtain highly purified CDP-ethanolamine in our laboratory nor have we been successful in securing a suitable compound from commercial sources. However we incubated ethanolamine-^{14}C with ATP and retinal homogenate to form phosphorylethanolamine-^{14}C. When CTP and fresh homogenate were added and the mixture was incubated for 45 min, we were able to isolate labeled phosphatidylethanolamine with an activity of 162 cpm/μg of lipid phosphorus. We tentatively conclude that phosphatidylethanolamine is synthesized in the retina by a mechanism very similar to a

TABLE 2 EFFECTS OF Mg^{++} ON LIPID SYNTHESIS AND THE INCORPORATION OF RADIOACTIVITY FROM CDP-CHOLINE-^{14}C INTO LECITHIN OF RAT RETINAL HOMOGENATE

$MgCl_2$	μg lipid phosphorus/g tissue	^{14}C Incorporated into Lecithin cpm/μg lipid phosphorus
μmoles/ml		
0	609 (6) ± 12	63
2.5	722 (6) ± 13	410
5.0 plus EDTA (10^{-4} M)	825 (6) ± 8	928
10.0 plus EDTA (10^{-4} M)	822 (6) ± 7	1518
10.0	819 (6) ± 7	—
20.0	587 (6) ± 11	1150
20.0 plus EDTA (10^{-4} M)	827 (6) ± 9	1537
0 (no CDP-choline)	514 (21) ± 29.8	—

The incubating medium and conditions of the experiment are those given in the Methods section with exceptions noted above.

mechanism for lecithin biosynthesis. The basic incubating medium used in these experiments was identical with that used with CDP-choline as precursor of lecithin.

Our value for the total lipid phosphorus content of normal rat retina (Table 3) is considerably lower than that found for calf retina by Broekhuyse (4). The standard error for the determinations attests to the wide variation in lipid phosphorus content from one group of animals to another. We found the distribution of various phosphatides in rat retina, expressed as percentage of total lipid phosphorus, to be as follows: lecithin, 40–50%; phosphatidylethanolamine, 30–40%; sphingomyelin, 5–10%; lysolecithin, 1.0%; phosphatidylserine and sphingomyelin (combined), 15–20%; and phosphatidylinositol, <5.0%.

Small quantities of CDP-choline-^{14}C are incorporated into lecithin by fractions of rod outer segments, but the incorporation can be accounted for entirely by activity from contaminating microsomes and mitochondria, as determined by cytochrome c oxidase and glucose-6-phosphatase assays.

In experiments in which the pigment epithelium was not included in the homogenate, activity of the mitochondrial and microsomal fractions was significantly

TABLE 3 COMPARISON OF NORMAL AND RCS RETINAE IN THEIR ABILITY TO INCORPORATE RADIOACTIVITY FROM CDP-CHOLINE-^{14}C INTO LECITHIN

	cpm/μg lipid phosphorus	Total Lipid Phosphorus μg/g tissue
Normal albino retinae (male)	1733	514 ± 29.8 (21)
RCS retinae (male)	1768	973 ± 13.4 (6)

The incubating medium consisted of the basic medium described in Methods. The numbers in parentheses represent the number of analyses.

reduced, with the microsomal fraction showing the greater loss of activity.

A concurrent study of certain metabolic mechanisms in the retina of a strain of rats suffering an hereditary retinal degeneration led us to include the retina of this animal in the present study. These animals are from a strain of piebald, agouti rat which undergoes a disease process closely resembling human retinitis pigmentosa. The phenotype of this animal is the same as that described by Dowling and Sidman (14) and by Sidman et al. (15) when the colony was at an earlier stage of inbreeding. The animals used in the experiments were approximately 1 yr old, and the appearance of the retina during dissection under magnification was that of a thick, fibrous band. The state of the disease process at this age would be that of Stages 4 and 5 as described by Bourne et al. (13). The rather violently distorted and altered retina of the RCS animal still contains the enzymes necessary to synthesize lecithin. Table 3 shows that while this abnormal retina contains an unusually high amount of phospholipid material, the amount of radioactivity from labeled intermediate incorporated into lecithin, on the basis of per gram of tissue, is no greater than in the normal, albino retina.

DISCUSSION

The results reported in the present communication indicate that the retina is capable of synthesizing lecithin and phosphatidylethanolamine by at least one pathway known to be in liver (10, 11) and brain (12). Retinal synthesis of lecithin appears to be mediated most rapidly by a particulate fraction obtained at 108,000 *g*. Our observations that retinal mitochondria also contain a system that incorporates CDP-choline into lecithin and that both mitochondrial and microsomal activity is reduced by removing the pigment epithelium are interesting in that both the inner rod segments and the pigment epithelium contain mitochondria, but the mitochondria of the inner segments are some distance from the lipid-rich rod disks (5, 6). We do not know at this time the source of the phospholipids of the outer rod segments, but we can postulate on the basis of our results that these compounds can originate from either the pigment epithelium or inner rod segments and that synthesis can be successfully carried out by the mitochondria or microsomes of these structures. While we have not completely characterized the transferases of retinal fractions, it is noteworthy that McCaman and Cook (44) found that phosphorylcholine–glyceride transferase of brain tissue appeared to be predominantly microsomal, but there was significant enzyme activity in the mitochondrial fraction. Thompson, Strickland, and Rossiter (45) used a highly purified preparation of mitochondrial membranes and gave conclusive evidence of phosphorylcholine–glyceride transferase activity in mitochondria. Akiyama and Sakagami (42) reported that phospholipids synthesized in one particulate fraction can be exchanged with those of another fraction. The structural integrity of the rod outer segments may well depend on a source of phospholipids from both the pigment epithelium and inner rod segments, with one of these structures being the more prominent supplier in the normal retina. It is possible that the source of phospholipids in the rod outer segments is controlled by conditions of oxygen supply, pH, or availability of precursors. The results obtained with added diglyceride reemphasize the importance of the nutritional status of the animal (7–9) in maintaining mechanisms and structures vital to the visual process.

Our observation of an abnormally high lipid content in the degenerated retina of the RCS (RD) animal was made at a time when the disease process in the animal included marked deterioration or absence of the visual cells and the appearance of extracellular lamellae between the pigment epithelium and the area of the rod outer segments; the bipolar and ganglion cells however, appeared to be intact (14). These observations suggest a metabolic defect of the pigment epithelium in the RCS animal. With the inner segments deteriorated, however, the integrity of the bipolar and ganglion cells remains a question.

With the unique experimental opportunities offered by the use of the RCS animal, the metabolic activity of the retina can be followed as individual layers deteriorate, although there is some overlapping of dystrophic characteristics from layer to layer in the time sequence of events.

The mapping of the metabolic pathways to the phospholipids of the retina and the characterization of the enzymes of the reactions involved could hopefully provide a means for the interpretation of certain reactions of the tissue to pharmacological agents as well as to provide a basis for the understanding of the participation of the phospholipids in the visual process.

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