Cytidine Metabolism in Photoreceptor Cells Of the Rat

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ABSTRACT During brief (30-min) incubations, isolated rat retinas accumulated [^{3}H]cytidine, converted it to cytidine triphosphate (CTP), and incorporated it into RNA and cytidine diphosphate-diacylglyceride (CDG), a phospholipid precursor of phosphatidylinositol (PI). Labeled CTP, RNA, and CDG contents were found to be two- to three-fold higher in photoreceptor cells than in cells of the inner retina. Autoradiograms showed that, within photoreceptor cells, silver grains representing RNA were concentrated over the nuclei in dark and light, while silver grains representing CDG were concentrated over the inner segments only after incubation in dark. The formation of labeled CTP and the synthesis of RNA were enhanced in light, while labeled CDG levels became reduced in light concurrent with an increase in the incoporation of labeled inositol into PI. The ^{3}H-labeled CDG content, however, was increased two- to fourfold in light in the presence of actinomycin D, and autoradiograms show a heavy concentration of silver grains over the inner segments of photoreceptor cells. These findings establish a role for cytidine nucleotides in photoreceptor cell metabolism and in light-dependent increases in RNA and PI synthesis. Furthermore, the observations indicate that a competition may exist in light for cytidine or CTP and suggest that availability of cytidine for CDG synthesis may have a regulatory role in PI metabolism within the photoreceptor cells.

Previous studies have demonstrated *de novo* synthesis of a cytidine-containing phospholipid precursor of phosphatidylinositol, cytidine diphosphate-diacyglyceride (CDG),[1] in isolated rat retinas (11). During brief (30-min) incubations, light stimulation was associated with enhanced synthesis (11) and turnover (12) of phosphatidylinositol (PI), synthesis of phosphatidic acid was also enhanced in light, while synthesis of CDG was not increased in light (11). Since CDG is a precursor for PI in the pathways of both synthesis and turnover, these studies suggested that formation of CDG from phosphatidic acid and cytosine triphosphate (CTP) may represent a rate-limiting step in PI metabolism.

Cytidine has also been shown to be used in vivo for RNA synthesis in photoreceptor cells of adult rat and frog retinas (4). Autoradiograms showed that 15 min after injection of [^{3}H]cytidine in the rat, labeled RNA was localized exclusively within the nuclei and at later times (14–24 h after injection) predominantly within the inner segments of photoreceptor cells (4).

The present study was done to measure the incorporation of tracer amounts of [^{3}H]cytidine into RNA and CDG concurrent with incorporation of [^{14}C]inositol into PI in isolated rat retinas incubated in dark or light. The relative amounts and localization of RNA and CDG synthesized from [^{3}H]-cytidine in dark and light and in the presence of actinomycin D were also evaluated by biochemical and autoradiographic techniques.

[1] *Abbreviations used in this paper:* CDG, cytidine diphosphate-diacylglyceride; CMP, cytidine monophosphate; CDP, cydine diphosphate; CTP, cytidine triphosphate; dpm, disintegrations per minute, PI, phosphatidylinositol; TCA, trichloroacetic acid.

MATERIALS AND METHODS

Materials: Pigmented Long-Evans rats were bred in our laboratory from stock purchased from Charles River Laboratories (Wilmington, MA). Puck's balanced salt solution was purchased from Grand Island Biological Co. (Grand Island, NY). Radioactive materials ([^{3}H]cytidine and [^{3}H]uridine) were purchased from ICN Radiochemicals, (Irvine, CA). [^{14}C]inositol, and Protosol solubilizer, and Econofluor and Formula 963 scintillation fluids were purchased from New England Nuclear (Boston, MA). Spectrophotometric grade solvents and reagent grade chemicals were obtained from Mallinckrodt Inc. (Paris, KY) and J. T. Baker Chemical Co. (Phillipsburg, NJ). autoradiography supplies and diisobutylketone were obtained from Eastman Kodak Co. (Rochester, NY). Polyethylenimide cellulose and silica gel 60 pre-coated plastic sheets were obtained from E. Merck Co. (Darmstadt, Federal Republic of Germany). Filters (EH, 0.5 μm) were obtained from Millipore Corp. (Bedford, MA). Phospholipid standards and actinomycin D, azacytidine, ribonuclease (RNase) Type A derived from bovine pancreas were purchased from Sigma Chemical Co. (St.Louis, MO) and from Supelco, Inc. (Bellefonte, PA).

Retina Incubation and Microdissection: The incorporation of

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tracer amounts of [^{3}H]cytidine (25 μCi/ml; 26 Ci/mmol; concentration of 10^{-6} M) was measured in retinas of 26- to 32-day-old pigmented Long-Evans rats dark-adapted for 64–88 h. The rats were killed and retinas were disssected and preincubated for 10 min in dim red light in ice-cold oxygenated Puck's bicarbonate buffer (Na^+, 142 mM; K^+, 4.4 mM; Ca^{2+}, 0.11 mM; Mg^{2+}, 0.76 mM; glucose, 6.1 mM; bicarbonate, 14.2 mM; and taurine, 0.05 mM). Incubations with radioactive cytidine were also done in the same buffer (designated as the standard incubation medium) in a rotating shaker (80 rpm) water bath at 37°C in an atmosphere of 95% oxygen and 5% CO_2 under a transparent Lucite cover. In some experiments, either actinomycin D or azacytidine were added to the incubation medium to determine the effects of these inhibitors of RNA synthesis on metabolism of [^{3}H]cytidine. In other experiments, retinas were incubated with [^{3}H]uridine (a precursor used exclusively for RNA synthesis) and the effects of actinomycin D were also determined. The dark-designated tubes were loosely wrapped and covered with tin foil while incubations in light were carried out in uncovered tubes exposed to a flashing white light stimulus (Grass PS-2 photostimulator, frequency of six cycles per second, each flash of 10-μs duration with average illuminance of 1,200 foot-candles).

After incubation, the retinas were rapidly washed free of radioactive medium in two 150-ml volumes of ice-cold 0.9% saline containing cytidine (1%) and formaldehyde (1–2%). After 1 to 2 min of wash, the retinas were frozen individually in liquid nitrogen, freeze-dried, weighed, and stored until analysis (5–8 d) at −80°C. In some experiments, after incubation, retinas were microdissected into photoreceptor cell and inner retina layers as previously described (11–13).

The uptake of [^{3}H]cytidine and the kinetics of uptake were measured by incubating retinas for 10 min in dark or light in media with 10–11 different concentrations of [^{3}H]cytidine (0.02×10^{-6} M–4.6×10^{-3} M). Retinas were washed, freeze-dried, weighed, placed in scintillation vials, solubilized with 1 ml of protosol, and counted in the presence of Econofluor.

In some experiments, retinas were incubated with [^{3}H]cytidine and [^{14}C]-inositol (10 μCi/ml; 250 mCi/mmol) to determine simultaneously the incorporation of these precursors into phospholipid in whole retinas and in microdissected photoreceptor and inner retina layers.

Biochemical Analyses: Incorporation of [^{3}H]cytidine into RNA, CDG, and conversion to cytidine monophosphate (CMP), cytidine diphosphate (CDP) and CTP within each whole retina or microdissected retinal sample were analyzed by microchemical techniques. Tissues were homogenized in 0.35 ml of 1 M KCl, sonicated, and the following aliquots were quickly removed and frozen at −80°C; 4 × 25 μl for trichloroacetic acid (TCA) precipitation, 100 μl for phospholipid extraction, and 50 μl for polyethylenimide cellulose chromatography. In some experiments tissues were homogenized in 0.4 ml of 1 M KCl and, in addition to the above, 50-μl aliquots of each were frozen for subsequent DNA analysis. Duplicate 15-μl aliquots were placed into scintillation vials and counted as a direct measure of total tissue radioactivity (expressed as dpm/mg dry weight or as dpm/whole retina).

Incorporation of [^{3}H]cytidine into RNA was analyzed by determining the radioactivity of hot-TCA-soluble material. One set of duplicate aliquots of each homogenate was treated with ice-cold TCA (4°C for 60 min) while another set was treated with hot TCA (90°C for 15 min followed by 60 min at 4°C); the precipitates were collected on filters (EH, 0.5 μM) and counted in the presence of Formula 963 aqueous scintillation cocktail. The difference between the radioactivity of the cold and that of the hot TCA precipitates (i.e., hot-TCA-soluble material) was taken as a measure of [^{3}H]cytidine incorporation into RNA.

Incorporation of [^{3}H]cytidine into CDG was quantitated in phospholipid extracts of retinas and microdissected photoreceptor cell and inner retina layers. Aliquots of homogenates (100 μl), brought to 0.5 ml and adjusted to contain 2 M KCl, were extracted with acidified solvent (chloroform:methanol:HCl, 124:65:1), and then with neutral solvent (chloroform:methanol:water, 86:14:1). The organic phases were pooled and purified with three consecutive washes as previously described (11, 12). The radioactivity of each extract was determined as a measure of [^{3}H]cytidine incorportion into phospholipid. Control experiments showed that this combination of solvents extracted 92–98% of the ^{3}H-labeled CDG from the tissues and that the radioactivity in the extract was due to [^{3}H]cytidine which was released upon alkaline hydrolysis (0.1 N NaOH, 30 min). Incorporation of [^{14}C]inositol into PI was determined from the fraction of total radioactivity associated with the PI spot after two-dimensional thin-layer chromatography.

Metabolites of [^{3}H]cytidine, CMP, CDP, and CTP were separated by polyethylenimide cellulose chromatography. To each 50-μl homogenate, a 25-μl mixture of cytosine, cytidine, and cytidine nucleotides in 0.1 N HCl was added (final concentration 10 mM with respect to cytidine, CMP, CDP, CTP, and cytosine). The homogenates were pelleted down by centrifugation at 4,000 *g* for 10 min, and 5-μl aliquots of the supernatant were spotted on (20 × 20 cm) polyethylenimide cellulose sheets prescored into eight lanes. The sheets were developed with four sequential solvents: twice, with 50 mM acetic acid, followed by double-distilled water, and then with 0.65 M LiCl with slight modifications of the method described by Honegger et al. (8). [^{3}H]Cytidine and metabolites were visualized under ultraviolet light; the spots were cut out, placed in vials, solubilized with 1.5 ml of 0.1 N HCl, and were counted in the presence of Formula 963 aqueous scintillation cocktail.

DNA content in 50-μl aliquots of homogenates of whole retina, photoreceptor cell, and inner retina layers was determined by a microadaptation of the method of Kissane and Robbins (9) as previously described (14).

The radioactivity for all experiments was measured in a Mark III liquid scintillation counter (Tracor Analytic Inc., Elk Grove Village, IL). The single-label counting efficiency for tritium was 60–68% and the double-label efficiencies for tritium and ^{14}C-radioactivity were 30–40% and 70–80%.

Autoradiography: Retinas were incubated for 30 min in dark or light with [^{3}H]cytidine (250 μCi/ml; 25 Ci/mmol). The incubations were terminated by the addition of fixative (2% formaldehyde in 0.1 M cacodylate buffer

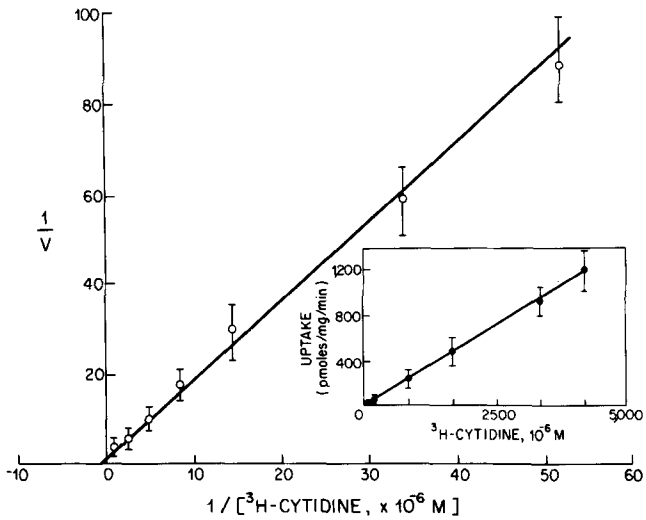

FIGURE 1 Double reciprocal plot of [^{3}H]cytidine uptake (V, pmol/mg retina dry weight/min) vs. cytidine concentration in the range of 0.02×10^{-6} M – 2×10^{-6} M). (*Inset*) Uptake of [^{3}H]cytidine vs. concentration of cytidine in the medium (10^{-6} M–4.6×10^{-3} M). The lines best fitted to each set of data points (by least square analysis) approach zero, indicative of a nonsaturable uptake mechanism for [^{3}H]cytidine in isolated rat retinas. The values represent the mean and the bars ± SD for four to six determinations.

TABLE 1

Labeled CTP, CDP, CMP, and Cytidine in Whole and Microdissected Rat Retinas after 30 Min of Incubation with [^{3}H]Cytidine in Dark or Light

Labeled compound	Whole retina	Photoreceptor layer	Inner retina
CTP			
Dark	19.6 ± 2	13.5 ± 1	4.3 ± 1
Light	40.2 ± 7	29.1 ± 4	8.2 ± 2
CDP			
Dark	17.7 ± 3	12.8 ± 2	5.6 ± 1
Light	31.5 ± 6	27.8 ± 4	9.2 ± 2
CMP			
Dark	8.8 ± 1	5.0 ± 1	3.2 ± 1
Light	8.6 ± 2	3.9 ± 1	3.2 ± 1
Free cytidine			
Dark	369.5 ± 58	225.1 ± 40	145.8 ± 20
Light	295.4 ± 50	185.5 ± 28	134.0 ± 22

Retinas were incubated for 30 min with [^{3}H]cytidine (25 μCi/ml; 26 Ci/mmol) in dark or light. Data are expressed as dpm × 10^{-3} per retina for whole retinas and as dpm × 10^{-3} per retina equivalent for photoreceptor and inner retina layers. These values were normalized so that the sum of total radioactivity within corresponding photoreceptor and inner retina layers was rendered equivalent to mean values obtained for whole retinas within each group. The values represent the mean ± SD for eight analyses.

TABLE II

Synthesis of ^{3}H-labeled RNA within Whole Rat Retina, Photoreceptor Cell Layer, and Inner Retina in Dark and Light

	[^{3}H]Cytidine incorporated			^{3}H-labeled RNA	
	Dark	Light	DNA	Dark	Light
	dpm/retina		*mg/retina*	*pmol/mg DNA*	
Retina	48,046 ± 7,100	91,288 ± 15,568	0.120 ± 0.02	7.0 ± 1.0	13.3 ± 2.6
Photoreceptor	39,946 ± 6,360	78,107 ± 11,340	0.088 ± 0.01	7.9 ± 1.2	15.5 ± 2.4
Inner retina	7,950 ± 1,256	13,181 ± 2,480	0.032 ± 0.005	4.4 ± 0.8	7.2 ± 1.4

Retinas were incubated for 30 min with [^{3}H]cytidine (26 Ci/mmol; 25 μCi/ml) in dark or light. The data for microdissected retina layers was normalized so that the sum of each corresponding photoreceptor cell and inner retina layer was rendered equivalent to values obtained for whole retinas both with respect to [^{3}H]cytidine incorporation and DNA content.

Assuming the DNA content of diploid retinal cells to be 7×10^{-12} g DNA (10), the rate of RNA synthesis in the photoreceptor cell layer compared to inner retina cells was 1.63 vs. 1.03×10^{-21} mol/cell/min. in dark and 3.62 vs. 1.84 in light.

The values represent the mean ± SD for six and eight determinations.

containing 3 mM calcium chloride, pH 7.2). Retinas were fixed for 10 min at room temperature followed by 30 min at 4°C with frequent changes of fixative. They were postfixed with 2% osmium tetroxide for 3 h on ice. Autoradiography was done on 0.5-μm sections using Ilford 5 or Kodak NTB-2 nuclear emulsion according to Gould and Dawson (6). The slides were examined after 4, 6, 8, and 11 d of exposure in a dark box kept at 4°C and were developed in Kodak Dektol. Some tissue sections on each slide were stained with toluidine blue while others were left unstained. Light microsope autoradiograms are presented (after 6-d exposure) in dark field using unstained sections and in phase contrast (light field) using adjacent sections stained with toluidine blue.

Loss and retention of free [^{3}H]cytidine or cytidine metabolites during fixation and dehydration were monitored by terminating the procedure at various stages and measuring the radioactivity retained within the retina and the radioactivity lost into the fixatives and buffers. In all experiments, four to six retinas were incubated together, in 0.6–1 ml of medium, one to two retinas were processed for autoradiography, three to five retinas were used to determine the distribution of radioactivity between unincorporated [^{3}H]cytidine, [^{3}H]-CDG, and [^{3}H]RNA. The sites of RNA synthesis were differentiated from those of CDG synthesis by comparison with retinas incubated with [^{3}H]uridine and by comparison with retinas from which CDG was extracted. To extract [^{3}H]-CDG, retinas incubated with [^{3}H]cytidine were carried through the fixation procedure and then were placed in acidified chloroform:methanol:HCl (124:65:1) solvent for 3 h at 22°C prior to osmication. The sites of ^{3}H-labeled CDG synthesis were determined in retinas that were incubated with [^{3}H]-cytidine, fixed, rinsed in buffer, and then digested with RNase (1 μg/ml Type A from bovine pancreas) for 3 h and 37°C (a treatment shown to hydrolyze RNA and to preserve CDG; see Table III) and in retinas incubated with [^{3}H]cytidine in the presence of actinomycin D (1 mg/ml), an inhibitor of RNA synthesis. The presence of ^{3}H-labeled CDG was confirmed by biochemical analysis, and the site of [^{3}H]CDG was identified by comparing autoradiograms of retinas incubated with [^{3}H]cytidine and actinomycin D to those incubated with [^{3}H]uridine and actinomycin D. Each of the autoradiography experiments was repeated three times.

RESULTS

Isolated rat retinas accumulated [^{3}H]cytidine by a nonsaturable mechanism in the concentration range of 0.02×10^{-6} M–4.6×10^{-3} M (Fig. 1). The uptake of [^{3}H]cytidine was similar in retinas incubated in dark or light and the data were pooled. Additional studies showed that uptake of [^{3}H]cytidine was linear for up to 45 min of incubation and that within 30 min of incubation, tissue-to-medium ratios were close to one. Table I shows that [^{3}H]cytidine accumulated in rat retinas was converted to CMP, CDP, and CTP. The levels of labeled CDP and CTP were higher in light while those of labeled CMP and free cytidine were similar in dark and light. ^{3}H-labeled CTP and CDP were two- to threefold higher within the photoreceptor cell layer than within the inner retina and were 2–2.4-fold higher in light compared with dark within both layers. The levels of CMP and free cytidine were similar in dark and light and were slightly higher in the photoreceptor cell layer compared with the inner retina.

Incorporation of [^{3}H]cytidine (26 Ci/mmol; 25 μCi/ml) into RNA during 30-min incubations was significantly higher

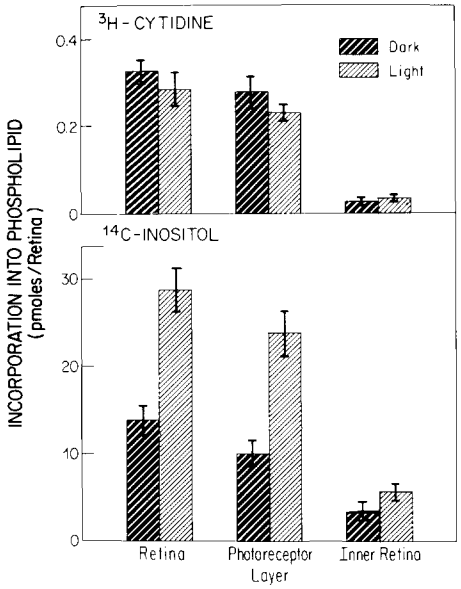

FIGURE 2 Incorporation of [^{3}H]cytidine (25 μCi/ml; 26 Ci/mmol) into CDG, measured simultaneously with incorporation of [^{14}C]-inositol (10 μCi/ml; 250 mCi/mmol) into PI in retinas incubated for 30 min in dark or light. Data for the microdissected layers have been normalized so that the sum of corresponding photoreceptor and inner retina layers was rendered equivalent to values obtained for whole retinas. The bars represent the mean and the lines within the bars ± SD for three to four analyses. Expressed in terms of DNA content (as a means of correcting for difference in cell numbers between the retinal layers), incorporation of [^{3}H]cytidine into CDG was about threefold higher in the phtoreceptor cell layer than in the inner retina (3.3 vs. 1.0 pmol/mg DNA in dark; and 2.8 vs. 0.94 in light). Incorporation of [^{14}C]inositol into PI was similar in dark (116 vs. 107 pmol/mg DNA) and was nearly twofold higher in photoreceptor cells compared to inner retina cells in light (268 vs. 145).

in light than in dark (Table II). The light vs. dark (L/D) ratios were, respectively, 1.9, 2.0, and 1.6 for the whole retina, the photoreceptor cell layer, and the inner retina. In terms of total retinal RNA synthesis, 82–86% of the [^{3}H]cytidine-labeled RNA was associated with the photoreceptor cell layer in dark and light. The higher values were not simply due to differences in cell numbers; expressed in terms of DNA content, ^{3}H-labeled RNA was 1.8-fold higher in dark and 2.2 times higher in light within the photoreceptor cell layer compared with the inner retina (Table II).

Incorporation of [^{3}H]cytidine and [^{14}C]inositol into phospholipid was measured simultaneously in double-label incu-

bations. Fig. 2 shows that while incorporation of [^{3}H]cytidine into CDG was lower in light ($P < 0.10$), incorporation of [^{14}C]inositol into PI was twofold higher in light than in dark incubations. Synthesis of CDG and PI was several-fold higher within the photoreceptor cell layer than within the inner retina. The light-dependent reduction in ^{3}H-labeled CDG and the light-dependent increase in ^{14}C-labeled PI are shown to be associated with the microdissected photoreceptor cell layer; [^{3}H]CDG was reduced by 21% ($P < 0.05$), and [^{14}C]PI was increased by 227% in light, while within the inner retina, [^{3}H]CDG was similar in dark or light, and [^{14}C]PI was only slightly higher in light than in dark (Fig. 2).

Dark- and light-field autoradiograms of representative retinas incubated with [^{3}H]cytidine (Fig. 3, *a* and *b*) show that radioactive grains were concentrated over nuclei of photoreceptor cells and over cells and nuclei in the inner nuclear layer in dark and light. After incubation in dark, photoreceptor inner segments contained a heavy density of radiolabeled silver grains that was comparable to the density of grains in the outer nuclear layer. In light, the density of radiolabeled

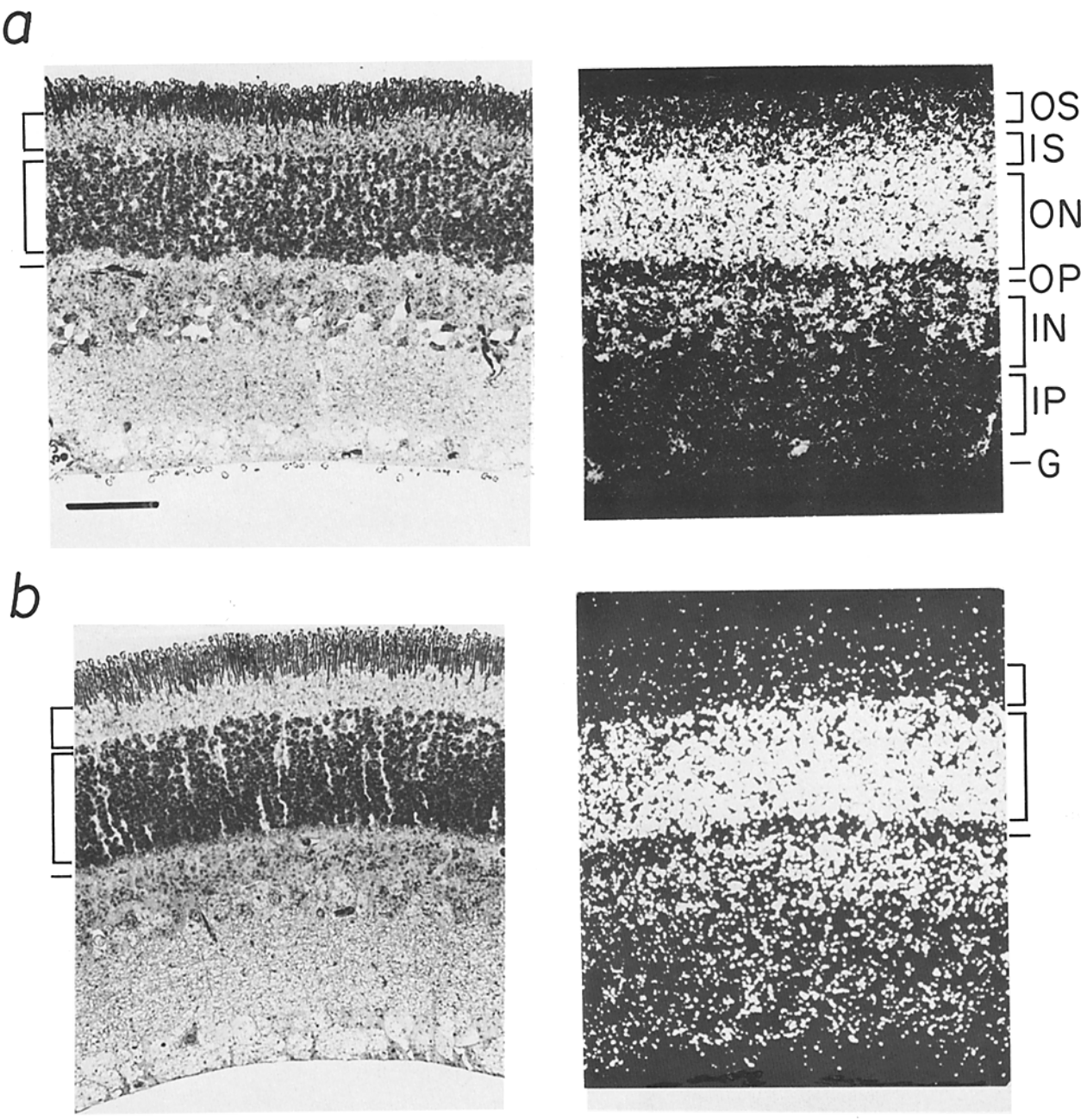

FIGURE 3 Representative autoradiograms of retinas incubated with [^{3}H]cytidine (250 μCi/ml; 26 Ci/mmol) in dark (*a*) or light (*b*). Autoradiograms were obtained after 6 d of exposure to photographic emulsion and were either in dark field (*right* side) using unstained sections or in phase contrast (lightfield; *left* side) using an adjacent section stained with toluidine blue. A concentration of silver grains was observed within the inner segment (*IS*) of photoreceptor cells only after incubation in dark (*a*). Radiolabeled grains were concentrated over nuclei in the outer (*ON*) and inner (*IN*) nuclear layers and were sparsely distributed over photoreceptor outer segments (*OS*), outer plexiform (*OP*), inner plexiform (*IP*), or ganglion cell (*G*) layers in dark or light. The brackets (*a* and *b*, *left* side, and *b*, *right* side) show the location of inner segments and outer nuclear layer, and the line designates the location of the outer plexiform layer. Bar, 50 μm. × 280.

grains within the photoreceptor inner segments was reduced compared to that within the outer nuclear layer and to that in dark. The distribution of radioactive grains within the outer and inner plexiform layers, ganglion cell layer, and photoreceptor outer segments was low in dark and light while within the inner nuclear layer the density of grains appeared to be higher in light compared with dark. In terms of total tissue radioactivity, the autoradiograms in Fig. 3, *a* and *b*, represent the localization of 20–25% free [^{3}H]radioactivity, 55–65% of ^{3}H-labeled RNA, and 15–20% ^{3}H-labeled CDG (Table III).

Fig. 4 shows representative light- and dark-field autoradiograms of a rat retina incubated with [^{3}H]uridine, a precursor used exclusively for RNA synthesis, in light. The pattern of radioactive grains was very similar to that obtained after incubation with [^{3}H]cytidine in light (compare with Fig. 3 *b*). The distribution of silver grains was most dense over photoreceptor nuclei in the outer nuclear and in the inner nuclear layers. Radioactive grains within the inner segments of photoreceptor cells were very sparse in dark or light. Retinas fixed after incubation with [^{3}H]uridine contained 15–25% free [^{3}H]uridine and 75–85% ^{3}H-labeled RNA.

Autoradiograms (Fig. 5) of retinas after RNase digestion and phospholipid extraction show that the distribution of radioactive grains after these procedures remained similar to that in untreated light-incubated retinas (Fig. 3 *b*), even though RNA or phospholipid and free [^{3}H]cytidine were effectively removed by these procedures. After RNase treatment, retinas contained 7% free ^{3}H radioactivity, 21% RNA, and 72% CDG and, after extraction with acidified solvent, 3% free radioactivity, 95% RNA, and 2% CDG (calculated from data in Table III). These biochemical analyses done in parallel with autoradiography demonstrate that cells in the outer and inner nuclear layers of the retina used [^{3}H]cytidine for both RNA and CDG synthesis.

Fig. 6, *a* and *b*, show representative autoradiograms of retinas incubated for 30 min in light with [^{3}H]uridine (Fig. 6 *a*), and [^{3}H]cytidine (Fig. 6, *b*) in the presence of actinomycin D. Retinas incubated with [^{3}H]uridine show very sparse labeling, while, in retinas incubated with [^{3}H]cytidine, radiolabeled silver grains were concentrated within the inner segments of photoreceptor cells (designated with a bracket). Biochemical analyses (Table IV), show that, in the presence of actinomycin D, CDG synthesis was enhanced nearly fourfold in light and RNA synthesis was reduced by 80% compared with standard incubations. In the presence of actinomycin D or azacytidine, incorporation of [^{3}H]cytidine into CDG was enhanced in light (1.4- and 2.3-fold, respectively). Biochemical analysis of retinas incubated as shown in Fig. 6 *b* or in repetitions of the same experiment show that, after incubation with [^{3}H]cytidine and actinomycin D in light,

TABLE III

Retention and Loss of [^{3}H]Cytidine during Autoradiography and the Distribution of Label in Fixed Rat Retinas

Treatment	Radioactivity lost during processing	Radioactivity retained in retina		
		Free [^{3}H]cytidine	RNA	CDG
Control retinas rinsed with saline	—	3,278 ± 416	749 ± 80	180 ± 30
Formaldehyde fixation	2,420 ± 296			
Buffer washes	425 ± 60			
Dehydration	46 ± 4	282 ± 40	760 ± 76	187 ± 32
RNase digestion after fixation	1,008 ± 210	22 ± 3	68 ± 9	228 ± 38
Extraction with solvent after fixation	660 ± 96	20 ± 3	698 ± 88	12 ± 2

Retinas were incubated for 30 min in light with [^{3}H]cytidine (26 Ci/mmol; 250 μCi/ml). The values represent the mean ± SD for four to eight analyses, and are expressed as dpm × 10^{-3} per whole retina.

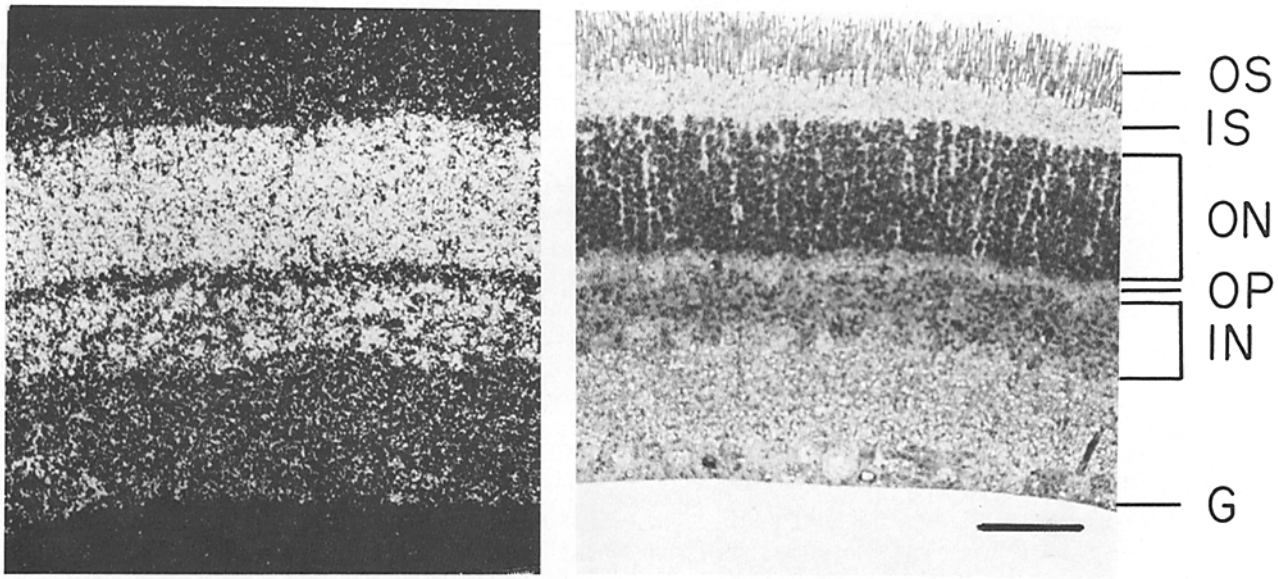

FIGURE 4 Representative dark- and light-field autoradiogram of a retina incubated for 30 min with [^{3}H]uridine (250 μCi/ml; 55 Ci/mmol) in light. Autoradigorams after incubation in dark or light were comparable; radioactive grains were concentrated over the outer and inner nuclear layers and none were associated with the inner segments. Bar, 50 μm. × 280.

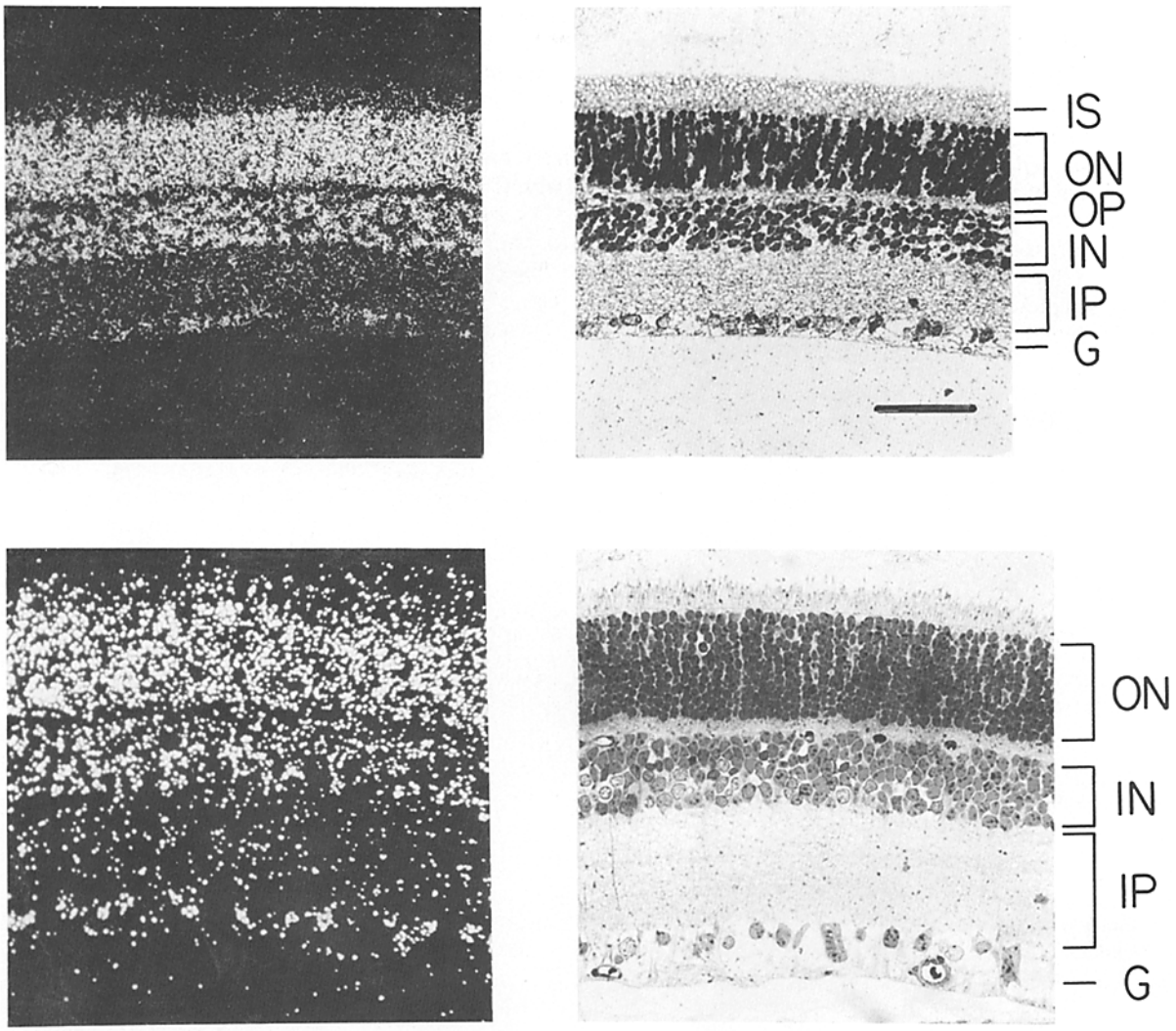

FIGURE 5 Representative dark- and light-field autoradiograms of retinas which, after incubation with [^{3}H]cytidine, either were extracted with acidified solvent (*top*) or were digested with RNase (*bottom*). Extracted retinas contained 95% [^{3}H]RNA, while RNase digested retinas contained 72% [^{3}H]CDG. Bar, 50 μm. × 280.

retinas contained ~65% [^{3}H]CDG, 15% [^{3}H]RNA, and 20% free [^{3}H]cytidine. Retinas incubated with [^{3}H]uridine and actinomycin D (Fig. 6 *a*) contained ~30% [^{3}H]RNA (<20% of that represented in Fig. 4) and 70% free [^{3}H]uridine. The levels of free [^{3}H]cytidine and [^{3}H]uridine were similar in these retinas. Comparison of the two autoradiogams (Fig. 6, *a* and *b*) and data in Table IV indicates that, in retinas incubated with [^{3}H]cytidine and actinomycin D, the dense labeling of the inner segments represents the site of enhanced [^{3}H]CDG synthesis.

DISCUSSION

These biochemical and autoradiographic studies demonstrate that, although all cells in the retina used [^{3}H]cytidine for both RNA and CDG synthesis, the utilization occurred to the greatest extent (80–85% of total retinal RNA and CDG synthesis) within the photoreceptor cell layer. During brief (30-min) incubations newly synthesized ^{3}H-labeled RNA was localized within nuclei, as shown previously (4), while [^{3}H]CDG was localized within the inner segments of the photoreceptor cells, a site previously associated with phospholipid synthesis in the frog retina (3). The high rates of RNA and CDG synthesis within the photoreceptor cell layer compared to inner retina were due in part to greater numbers of cells and in part to more rapid rates of synthesis; based on DNA content, RNA and CDG synthesis were two- to three-fold higher in photoreceptor cells than in cells of the inner retina.

Light stimulation was associated with enhanced incorporation of [^{3}H]cytidine into RNA throughout the entire retina but particularly within the photoreceptor cell layer. This observation is consistent with a previous report that showed enhanced incorporation of labeled uridine in light in frog photoreceptor cells (7). RNA synthesis has been previously shown to be enhanced with visual stimulation in specific areas (forebrain roof) of chick brain (1) and with behavioral training in the hippocampus of rats (5). In the latter instance, the newly synthesized RNA was shown to be enriched with respect to messenger RNA. At present, it is not possible to state which particular classes of RNA were enhanced in light in the photoreceptor cells in the rat.

Fig. 7 represents a model for cytidine metabolism in the isolated rat retina based on the present data and other studies (11, 12). Cytidine is shown to be converted to CMP, CDP, and CTP; the latter is then mainly used in the two divergent

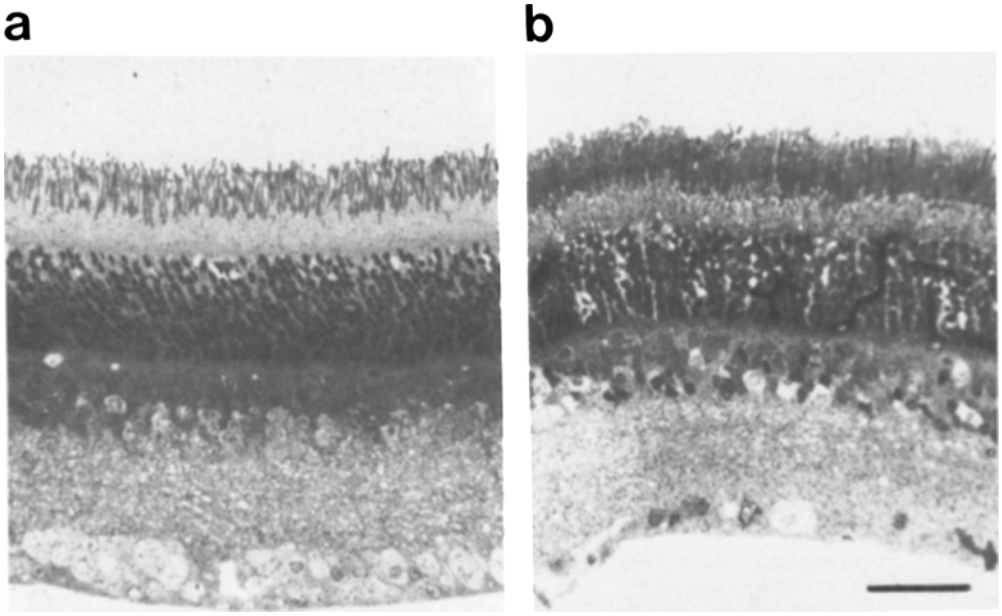

FIGURE 6 Representative autoradiograms of retinas incubated in light with [^{3}H]uridine (*a*) or [^{3}H]cytidine (*b*) in the presence of actinomycin D. The heavy density of silver grains within photoreceptor cell inner segments (bracketed area) designates the site of [^{3}H]CDG synthesis in retinas incubated with [^{3}H]cytidine and Actinomycin D. Bar, 50 μm. × 280.

TABLE IV

Effects of Actinomycin D and Azacytidine on the Incorporation of [^{3}H]Cytidine into RNA and CDG in Isolated Rat Retinas

	Dark		Light	
	RNA	CDG	RNA	CDG
Standard incubation	0.84 ± 0.14	0.30 ± 0.05	1.60 ± 0.32	0.25 ± 0.04
Actinomycin D	0.28 ± 0.03	0.70 ± 0.15	0.30 ± 0.07	0.96 ± 0.16
Azacytidine	0.58 ± 0.10	0.30 ± 0.08	0.49 ± 0.10	0.69 ± 0.12

Retinas were incubated for 30 min in dark or light in the standard incubation medium ([^{3}H]cytidine 25 μCi/ml; 26 Ci/mmol) and in the presence of actinomycin D (1 mg/ml) or azacytidine (1 mg/ml). The data are expressed as pmol [^{3}H]cytidine incorporated into RNA or CDG, per retina.
The values represent the mean ± SD for six to eight determinations.

pathways of RNA and CDG synthesis. The heavy arrows denote reactions shown to be enhanced in light; these include the formation of CDP and CTP, RNA synthesis, and the synthesis and hydrolysis of PI. Synthesis of DNA has been considered negligible in nondividing retinal cells (4), and the formation of CDP-choline and CDP-ethanolamine have not been investigated. Incorporation of [^{3}H]cytidine into CDG was reduced in light within the photoreceptor cell layer, and concurrently incorporation of [^{14}C]inositol into PI was enhanced. These observations indicate that CDG may be limiting for PI synthesis in light, and suggest that the formation of CDG from CTP and phosphatidic acid may represent a rate-limiting step in PI synthesis in light within photoreceptor cell inner segments as CTP is used preferentially for RNA synthesis within photoreceptor cell nuclei. This idea is supported by the finding that light-enhanced incorporation of [^{3}H]cytidine into CDG within photoreceptor cell inner segments was observed only when RNA synthesis was inhibited by actinomycin D.

These studies establish a role for cytidine nucleotides in photoreceptor cell metabolism and in light-dependent increases in RNA and PI synthesis. CTP has been previously shown to be present in photoreceptor cells (2), and utilization of [^{3}H]cytidine for RNA synthesis has been associated with photoreceptor cell nuclei (4), while utilization of cytidine for CDG synthesis has not been previously demonstrated in the

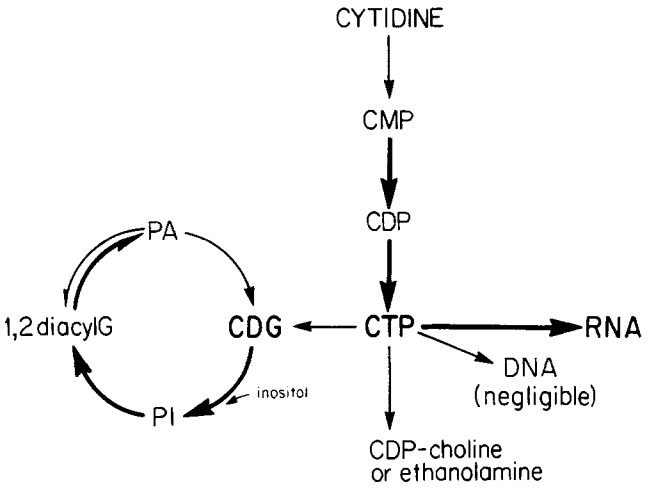

FIGURE 7 A model for cytidine metabolism in isolated rat retinas based on present findings and other studies (11, 12). The heavy arrows denote reactions shown to be enhanced in light; these include the formation of CDP, CTP, synthesis of RNA, conversion of CDG to PI, hydrolysis of PI to 1,2 diacyglycerol (1,2 diacyl G) and resynthesis of PI via formation of PA from 1,2 diacyl G and ATP. Light-enhanced synthesis of CDG (from PA and CTP) was detected only when RNA synthesis was inhibited by actinomycin D or azacytidine.

retina. Furthermore, the present findings suggest that the availability of cytidine as CTP for CDG synthesis may have a regulatory role in PI metabolism within the photoreceptor cells.

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