Affinity Labeling of Lecithin Retinol Acyltransferase†

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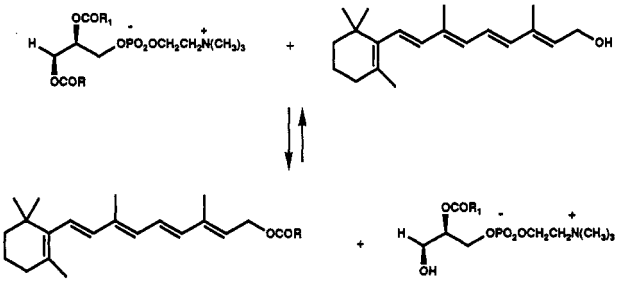
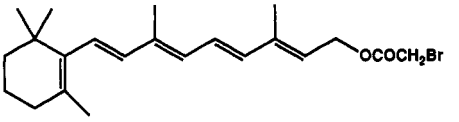
ABSTRACT: Lecithin retinol acyltransferase (LRAT) transfers acyl groups regiospecifically from the *sn*-1 position of lecithins to *all-trans*-retinol (vitamin A) and similar retinoids. LRAT is essential for the biosynthesis of 11-*cis*-retinal, the visual pigment chromophore. LRAT is also required for the general dietary mobilization of vitamin A. The enzyme is membrane-bound and has been solubilized and partially, but not completely, purified. It is demonstrated here that *all-trans*-retinyl α-bromoacetate (RBA) is a potent irreversible affinity labeling agent of LRAT. The measured K_I = 12.1 μM and the pseudo-first-order rate constant for inhibition is $k_{inh} = 8.2 \times 10^{-4}$ s^{-1}. The specificity of the inhibition process is further evidenced by the observation that α-bromoacetate derivatives of hydrophobic alcohols which are not substrates for LRAT, such as cholesterol and β-ionol, are not inhibitors of the enzyme. Labeling of the partially purified enzyme with ^{3}H-RBA showed a single radiolabeled band of molecular weight approximately 25 000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Lecithin retinol acyltransferase (LRAT)[1] catalyzes the regioselective transfer of acyl groups from the *sn*-1 position of phospholipids to *all-trans*-retinol (vitamin A), generating retinyl esters (MacDonald & Ong, 1987; Barry et al., 1989; Saari & Bredberg, 1989). The regioselectivity for the *sn*-1 acyl group makes LRAT thus far unique, and possibly a member of a new class of acyltransferases. The overall reaction sequence is shown in Scheme I. This membrane-bound enzyme is specific for phosphatidylcholine derivatives (lecithin) and for retinol analogs (Cañada et al., 1990). LRAT has so far been determined to be present in the intestine, liver, and retinal pigment epithelium (RPE) (Ong et al., 1991; MacDonald & Ong, 1987; Barry et al., 1989; Saari & Bredberg, 1989). LRAT has been implicated in the mobilization of vitamin A in the liver and intestine. LRAT also plays an essential role in visual pigment regeneration, since *all-trans*-retinyl esters are the "high-energy" precursors for the isomerohydrolase which generates 11-*cis*-retinol in the RPE (Deigner et al., 1989). Oxidation of 11-*cis*-retinol in the RPE produces the visual chromophore 11-*cis*-retinal. In a sense, LRAT is an energy transducer in the RPE, linking the chemical potential of phospholipids to the formation of the thermodynamically unstable visual chromophore (Rando, 1991).

LRAT, which has been solubilized and partially purified (Barry et al., 1989), functions by a kinetic mechanism involving an ordered ping-pong bi-bi mechanism (Shi et al., 1993). The enzyme is first acetylated by the lecithin to generate an acyl enzyme intermediate and a 2-acyllysophospholipid. The vitamin A binds and is then esterified to generate the retinyl ester and the free enzyme. Other mechanistic or structural information on the enzyme is lacking. However, it is known that LRAT can be inactivated by a series of sulfhydryl-directed reagents, including *p*-hydroxymercuribenzoate, implying that the enzyme is a sulfhydryl-dependent enzyme (Fulton & Rando, 1991). Previously, we had shown that the enzyme is powerfully inhibited by *all-trans*-retinyl α-bromoacetate (RBA) (Trehan et al., 1990). Here we show that RBA is an active-site-directed irreversible inhibitor of the enzyme. By use of radiolabeled ^{3}H-RBA, an approximately 25-kDa protein is identified. This labeled protein is likely to be LRAT itself or its catalytic subunit.

Scheme I: Transesterification of Vitamin A Catalyzed by LRAT





all-trans-retinyl α-bromoacetate

MATERIALS AND METHODS

Materials

Frozen bovine eye cups were obtained from W. L. Lawson Co. (Lincoln, NE). [11,12-^{3}H$_2$]*All-trans*-retinol (2.34 Ci/mmol) was obtained from Dupont–New England Nuclear. *All-trans*-retinol, bovine serum albumin (BSA), dithiothreitol (DTT), and L-α-dipalmitoylphosphatidylcholine (DPPC) were from Sigma Inc. α-Bromoacetyl chloride was from Aldrich Inc. Triton X-100 was from Calbiochem Corp. Disodium

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[1] Abbreviations: LRAT, lecithin retinol acyltransferase; RBA, *all-trans*-retinyl α-bromoacetate; DPPC, dipalmitoylphosphatidylcholine; IBA, β-ionylidene bromoacetate; IEBA, β-ionylidenethanol bromoacetate; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; RPE, retinal pigment epithelium; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

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ethylenediaminetetraacetate (EDTA·2Na) was from Fisher Scientific Co.

Methods

Syntheses. RBA, IBA, and IEBA were prepared by published procedures (Gawinowicz & Goodman, 1982). Cholesterol bromoacetate was prepared in an identical manner. [11,12-$^3H_2$]*All-trans*-retinyl α-bromoacetate (specific activity = 2.34 Ci/mmol) was prepared in the same way starting with [11,12-$^3H_2$]*all-trans*-retinol (specific activity = 2.34 Ci/mmol). The radioactive product was dissolved in a solution of 7% dioxane–hexane and purified by HPLC (Waters 625LC system, Lichrosorb Si-60, 7% dioxane–hexane). The retention time of the compound was 3.0 min and the yields of the purified compound ranged between 15% and 25%. The concentration of RBA was determined on a UV/vis spectrophotometer (Perkin-Elmer λ 3B) at a wavelength of 325 nm (ϵ = 47 000).

Partial Purification of Lecithin Retinol Acyltransferase. The method used for the partial purification of LRAT was substantially the same as previously reported (Barry et al., 1989). Bovine pigment epithelium membranes were solubilized in 20 mM Tris-HCl, pH = 9.0, 2 mM DTT, 1 mM EDTA·2Na, 1% Triton X-100, and 1 mg/mL DPPC. After thorough mixing for 1 h at 4 °C, the solubilized material was centrifuged at 4 °C at 10500*g* for 1 h. The supernatant was applied to a Mono-Q column on a LKB-Pharmacia Biotechnology Inc. Superose 6 LCC-500 FPLC system at 5 °C and eluted using a linear gradient of buffers A and B (buffer A, 20 mM Tris-HCl, pH = 9.0, 2 mM DTT, 1 mM EDTA·2Na, 0.1% Triton X-100, and 0.1 mg/mL DPPC; buffer B, buffer A plus 1 M NaCl). The collected fractions were assayed as previously published (Barry et al., 1989). The fractions were assayed and stored at −70 °C separately. Enzyme partially purified in this manner (specific activity = 1.3 μmol min^{-1} mg^{-1}) is stable for months at −20 °C. Protein concentrations were determined by the Peterson modification of the Lowry method (Peterson, 1977).

Inhibition of LRAT by RBA. All experiments were carried out under dim red light to avoid isomerization of the retinoids. The activity of LRAT was determined by incubating LRAT with dipalmitoylphosphatidylcholine (DPPC) (400 μM) and [11,12-$^3H_2$]-*all-trans*-retinol (2.34 Ci/mmol, 0.2 μM) at 23 °C in Tris-HCl (170 mM, pH = 8.0) and dithiothreitol (3.3 mM). After a 10-min incubation, 500 μL of methanol was added to stop the reaction. After thorough mixing, 100 μL of water and 400 μL of hexane were added. The mixture was again agitated by vortexing for 1 min and centrifuged for 5 min at 15 °C. Finally, 200-μL aliquots of the hexane layer were removed for analysis as previously described (Barry et al., 1991).

Partially purified LRAT (9.5 μL, 27 mg of protein/mL) was diluted with 170.5 μL of Tris-HCl (170 mM, pH = 8.0) and dithiothreitol (3.3 mM) at 23 °C. HPLC-purified RBA (0.15 nmol) was dissolved in 20 μL of BSA (2.5% in distilled H_2O) and added to the buffer solution containing LRAT. The final concentrations of RBA and LRAT were 0.75 μM and 1.28 mg of protein/mL, respectively. Inhibition was measured at 0, 10, 20, 30, and 40 min by taking 10-μL aliquots of the incubation mixture and adding them to the assay mixture containing DPPC, ^{3}H-*all-trans*-retinol, 70 μL of buffer, and 20 μL of the BSA solution. The final concentrations of DPPC, ^{3}H-*all-trans*-retinol, LRAT, and RBA were 400 μM, 0.2 μM, 0.128 mg of protein/mL, and 0.75 μM, respectively. Since RBA slowly decomposes with time in the buffer solution, the levels of RBA were monitored as a function of time by HPLC analysis to determine the actual RBA concentrations. The conversion of retinol to retinyl palmitate was analyzed on a normal-phase HPLC column (Dynamax-60A) connected to an on-line Berthold radioactivity monitor and eluted with hexane/ethyl acetate/2-propanol (90/10/1) at a flow rate of 1.5 mL/min.

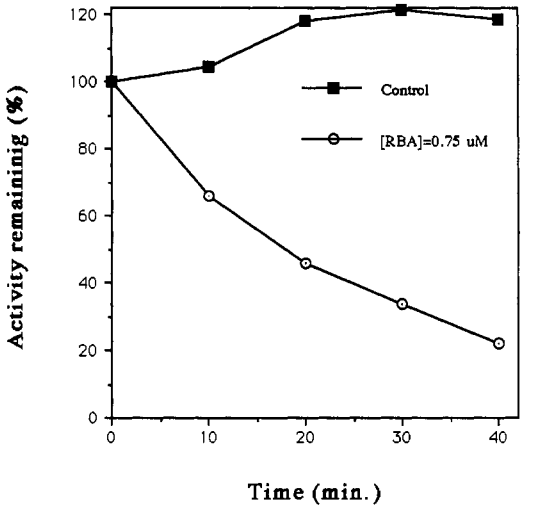


FIGURE 1: Time course for the inhibition of LRAT by RBA. These experiments were performed as indicated in the Methods section. The observed inhibition at 10, 20, 30, and 40 min was 34 ± 2%, 54.1 ± 8%, 66.4 ± 1%, and 78.1 ± 2% respectively. Control experiments were also performed by incubating LRAT for 0, 10, 20, 30, and 40 min in the absence of RBA. Triplicate assays were performed in all cases. The average of these values is shown in the figure.

RESULTS

Initial experiments were aimed at establishing a time course for the inhibition of LRAT by RBA which would imply an irreversible mode of inhibition. These experiments were performed by incubating LRAT with RBA and then removing aliquots of preparation and diluting them into assay buffer containing radioactive substrate. The RBA is diluted enough in the assay buffer so that further inhibition is effectively quenched. As shown in Figure 1, a time-dependent mode of inhibition could be established which suggests an irreversible mode of inhibition. Further experiments unequivocally demonstrated that the enzyme was irreversibly and covalently inhibited. First, UV irradiation of RBA at 365 nm results in its rapid photochemical destruction without affecting LRAT activity. Incubation of partially purified LRAT with a preirradiated solution of RBA did not result in the inhibition of the enzyme. Treatment of LRAT with 5 mM RBA to achieve total inactivation followed by irradiation at 365 nm did not cause reactivation of the enzyme. Finally, treatment of another sample of inactivated enzyme with Bio-Beads and 1% BSA—conditions which remove excess RBA—did not result in the reactivation of LRAT. Thus, once inhibited, LRAT cannot be reactivated by the removal of excess RBA.

Although RBA is a substrate analog and inactivates LRAT at relatively low concentrations, it must still be demonstrated that the observed inhibition is specific. One way to establish specificity of inhibition is to demonstrate that the mode of inhibition is saturable. The simple kinetic scheme shown below can be derived to describe a specific mode of inhibition (Kitz & Wilson, 1964). When this scheme was applied to the inactivation of LRAT by RBA, the results shown in Figure 2 were obtained. As can be seen from the inverse plot in Figure 2B, saturation is observed. The measured K_I = 12.1 μM and the first-order rate of inhibition is k_{inh} = 8.2 × 10^{-4}

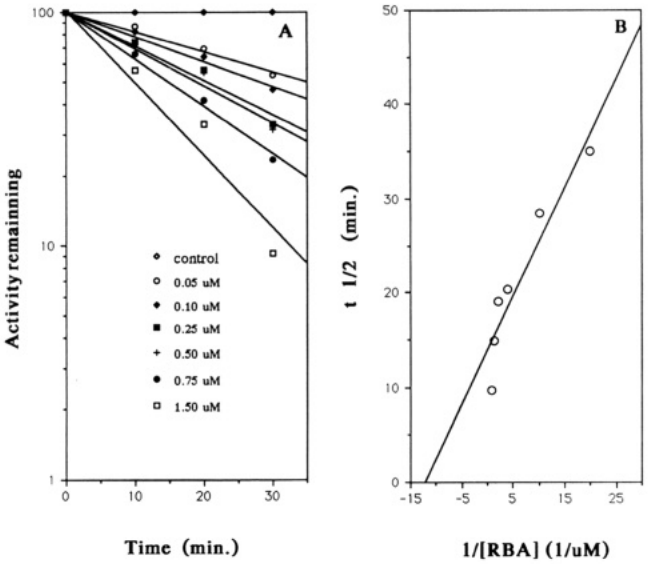


FIGURE 2: First-order kinetics of the irreversible inhibition of LRAT by RBA. The experiments were performed as described for Figure 1 and in the Methods section. The incubation times for inhibition were 0, 10, 20, and 30 min, and the concentrations of RBA were 0.05, 0.10, 0.25, 0.50, 0.75, and 1.50 μM respectively. In panel A, pseudo-first-order plots for the inhibition are shown, and inverse plots are shown in panel B. The determined $K_I = 12.1 \pm 1.8$ μM and $k_{inh} = (8.2 \pm 0.9) \times 10^{-4}$ s^{-1} at 23 °C. The kinetic equation used is shown in the Results section.

s^{-1}. Had RBA inactivated LRAT by a simple chemical alkylation mechanism, saturation would not have been observed, as the kinetics of LRAT inactivation would have been second order.

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} EI \xrightarrow{k_{inh}} E\text{-}I$$

E: active enzyme; I: inhibitor; EI: reversible enzyme inhibitor complex; E-I: covalently modified enzyme

Assuming that $[I] >> [E]$ and $[EI]$ is constant,

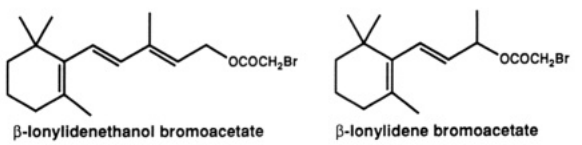
$E_0 = [E] + [EI] + [E\text{-}I]$, $[I][E] / [EI] = K_I$,

$-d([E] + [EI]) / dt = -d[E] / dt = k_{inh}[EI]$, then $-dE / dt = k_{inh}\, dt / (1 + K_I / [I])$

and after integration: $\ln (E / E_0) = -k_{inh}\, t / (1 + K_I / [I])$

or $t_{1/2} = 0.69 / k_{inh} (1 + K_I / [I])$ [$t_{1/2}$: the time at which E/E_0 equals 0.5]

To probe the specificity of the inactivation process, other hydrophobic alkylating agents, in addition to RBA, were studied as possible inactivators of the enzyme. Neither β-ionylidene bromoacetate (IBA) (Gawinowicz & Goodman, 1982) nor β-ionylidenethanol bromoacetate (IEBA) (Gawinowicz & Goodman, 1982) appreciably inhibited (<10%) the enzyme after incubation of the enzyme with 10 μM inhibitor for 10 min. Similar results were obtained with



cholesterol α-bromoacetate and cholesterol α-chloroacetate. Neither of these compounds was capable of measurably inactivating LRAT when incubated with the enzyme for 10 min at a concentration of 10 μM. These results demonstrate that the observed inactivation of LRAT by RBA is not due to a nonspecific alkylation mechanism dependent on a hydrophobic alkylating agent.

FIGURE 3: Labeling of LRAT by ^{3}H-RBA. A solution of detergent-solubilized and partially purified LRAT (2.0 mL, 5.13 μg/mL) was incubated with 10 mM cholesterol bromoacetate for 10 min at 23 °C to block cysteine residues other than those at the active site of LRAT. Radioactive RBA ([11,12-$^3H_2$]-*all-trans*-retinyl α-bromoacetate; 1.2 μM, specific activity = 2.34 Ci/mmol) was added, and the incubation was allowed to proceed for 15 min. Assay of the enzyme showed that 20% had been inhibited. The solution was then concentrated on a Centricon-30 to approximately 50 μL at 4 °C and then precipitated by adding 1.5 mL of chilled acetone(–20 °C). After centrifugation at 14 000 rpm (4 °C), the pellet was suspended in 200 μL H_2O and the proteins were precipitated with trichloroacetic acid and sodium deoxycholate according to the method of Peterson (1977). The precipitate was washed twice with mL of chilled acetone and loaded on a 3.0% polyacrylamide stacking gel followed by a 12.5% gel, and was subsequently stained with Coomassie Brilliant Blue. Lane 1, 10 μL of Bio-Rad prestained SDS–PAGE low molecular weight standards [phosphorylase B (106 000), BSA (80 000), ovalbumin (44 500), carbonic anhydrase (32 500), soybean trypsin inhibitor (27 500), and lysozyme (18 500)]. The lowest molecular weight standard is at the bottom of the figure proceeding to the highest at the top. Lane 2, 10.2 mg of partially purified LRAT. Lane 3, autoradiograph of lane 2, exposed for 8 days.

LRAT has been exceedingly difficult to purify to homogeneity, and consequently the molecular identification of this important enzyme has been lacking. Since RBA is a potent and specific irreversible inhibitor of the enzyme, it should be possible to specifically label LRAT with radioactive RBA and thus identify the enzyme. ^{3}H-RBA was prepared as indicated in the Methods section and incubated with partially purified LRAT. The enzyme preparation was preincubated with nonradioactive cholesterol α-bromoacetate to block nonspecific sites potentially labeled with ^{3}H-RBA. The results of this experiment are shown in Figure 3. The second lane shows the Coomassie blue staining profile for the partially purified LRAT, and the third lane shows the autoradiogram using the labeled RBA. There is only one major protein labeled, of molecular weight approximately 25 000. Molecular weight standards are shown in the first lane.

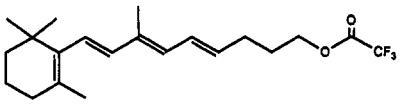
DISCUSSION

It had previously been demonstrated that RBA is a potent inhibitor of LRAT (Trehan et al., 1990). Using this inhibitor, it was possible to demonstrate that vitamin A esterification by LRAT action is an essential step in the biosynthesis of 11-*cis*-retinoids in the visual system (Trehan et al., 1990). This observation that RBA could completely abolish the processing of vitamin A by LRAT suggested the possibility of an irreversible mode of inhibition. In early studies using the crude enzyme preparations then on hand, it appeared that the mode of inhibition was reversible (Trehan et al., 1990), but now, using a detergent-solubilized purified enzyme

preparation, it is shown that LRAT is in fact irreversibly inactivated by RBA, as we had originally suspected.

Several initial criteria were used to demonstrate irreversibility. First, inhibition proved to be time-dependent. Second, the activity of the inhibited enzyme could not be restored by removing excess RBA. The time-dependent loss of LRAT activity in the presence of RBA was pseudo-first-order and saturable, with a measured $K_I = 12.1$ μM and a $k_{inh} = 8.2 \times 10^{-4}$ s^{-1}. This observation is entirely consistent with an active-site, affinity labeling mode of inhibition and demonstrates that the inactivation process is specific in nature and not due to random alkylation of the enzyme. This latter point is also borne out by the findings that LRAT is not appreciably inactivated by cholesterol bromoacetate, IBA, or IEBA. Since these reagents contain the same alkylating functionality as RBA, their failure to inhibit LRAT is consistent with the notion that the inactivation of LRAT by RBA is by a specific affinity labeling mechanism. It is noteworthy that neither cholesterol nor β-ionol is a substrate for LRAT (Cañada et al., 1990). Interestingly, IBA and IEBA, but not RBA, irreversibly inhibit human plasma retinol-binding protein (Gawinowicz & Goodman, 1982).

It is interesting to note that the binding constant of RBA to LRAT, which is 12.1 μM, is considerably lower than those of other *all-trans*-retinyl esters, such as *all-trans*-retinyl palmitate (Shi et al., 1993). Retinyl esters of the latter type are exceedingly weak inhibitors of LRAT (Shi et al., 1993). In fact, inhibition of LRAT by *all-trans*-retinyl palmitate cannot be measured even at 100 mM concentrations of the latter analog. The enhanced binding of RBA to the enzyme suggests that a special mechanism may be involved. Previously we had shown that 13-desmethyl-13,14-dihydro-*all-trans*-retinyl trifluoroacetate (**1**) is a potent reversible inhibitor of LRAT, with a K_I of 11.4 ± 2.8 μM (Shi et al., 1993). The



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corresponding acetate derivative of **1** is not measurably an inhibitor of LRAT. Moreover, trifluoroketone analogs of retinoids similar to **1** are also potent inhibitors of the enzyme (I. Hubacek and R. R. Rando, unpublished results). That the various electron-withdrawing retinoid analogs are potent inhibitors of LRAT suggests that they are possibly transition-state analogs.

As mentioned before, the kinetic mechanism of LRAT action involves a ping-pong bi-bi mechanism in which the lecithin first reacts with the enzyme, transferring an acyl group to an active-site nucleophilic amino acid (Shi et al., 1993). After the lysolecithin departs, the vitamin A binds and the acyl group is transferred to it. Transition-state structures are likely to involve tetrahedral intermediates. It is entirely possible that the haloester inhibitors either bind to the active-site nucleophile via a covalent tetrahedral complex or form hydrates which mimic transition-state tetrahedral structures. Alkylation of an active-site nucleophile by RBA presumably would occur after complex formation. Since LRAT is quite sensitive to sulfhydryl-directed reagents, such as *p*-hydroxymercuribenzoate, a catalytically essential active-site cysteine residue would be an obvious candidate for the site of alkylation by RBA.

Although LRAT is readily solubilized and partially purified, its total purification has not been achieved. As is typical of many membrane-bound enzymes, enzyme denaturation occurs as purification proceeds. Therefore, the use of a specific affinity labeling agent to identify the catalytic polypeptide is important for characterizing the enzyme, producing antibodies to it, and eventually cloning it and determining its primary sequence. The labeling of partially purified LRAT with ^{3}H-RBA allowed the identification of a single polypeptide of molecular weight ~25 000 by SDS–PAGE. This protein is likely to be either the holoenzyme or the catalytic subunit of LRAT (if the enzyme is multisubunit in nature). Further characterization of this labeled protein is currently underway. It will be of interest to determine the sequence of this enzyme, because it is the first lecithin-dependent acyltransferase which is regiospecific for the *sn*-1 position of the phospholipid. A comparison of the active-site sequence to that of lecithin cholesterol acyltransferase (LCAT) will also be of interest. LCAT is a soluble ~50-kDa enzyme which transfers an acyl group regiospecifically from the *sn*-2 position of lecithin to cholesterol (Francone & Fielding, 1991). It is not unlikely that the two enzymes are mechanistically similar. Finally, the observation that RBA specifically labels LRAT opens up the possibility that RBA and similar reagents can be used to tag and identify novel lecithin acyltransferase enzymes similar to LRAT.

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