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Substrate Specificities and Mechanism in the Enzymatic Processing of Vitamin A into 11-*cis*-Retinol†

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ABSTRACT: The biosynthesis of 11-*cis*-retinol in the retinal pigment epithelium requires two consecutive enzymatic reactions. The first involves the esterification of *all-trans*-retinol by lecithin retinol acyltransferase (LRAT). The second reaction involves the direct conversion of an *all-trans*-retinyl ester into 11-*cis*-retinol by an isomerase-like enzyme. This latter reaction couples the free energy of hydrolysis of an ester to the thermodynamically uphill trans to cis conversion, thus providing the energy to drive the latter process. In this paper both enzymes are studied with respect to their substrate specificities to provide information on mechanism. The isomerase is shown to be highly specific with respect to the ionylidene ring system and substitution at C15, whereas sterically bulkier substituents at C9 and C11 are permitted. C5 and C13 demethyl retinoids are isomerized, removing from consideration isomerization mechanisms involving C-H abstraction at the C5 or C13 methyl groups of the retinoid. On the other hand, C9 demethyl retinoids are not isomerized. A C-H abstraction mechanism is unlikely at the C9 methyl group as well, because no kinetic deuterium isotope effect is found with *all-trans*-19,19,19-trideuterioretinoids and isomerization of unlabeled retinoids occurs without the incorporation of deuterium when the isomerization is performed in D_2O. LRAT proved to be broadly specific for retinols but was relatively inert with other hydrophobic alcohols including cholesterol. The enzyme is also highly specific for phosphatidylcholine analogues versus other potential membranous acyl donors such as phosphatidylethanolamine and phosphatidylserine.

Vision begins with the photoisomerization of the 11-*cis*-retinal Schiff base chromophore of rhodopsin to its *all-trans*-retinal congener (Hubbard & Wald, 1952). *all-trans*-Retinal is reduced in the rod outer segments to *all-trans*-retinol (vitamin A) and carried off to the pigment epithelium where it is esterified and isomerized to produce 11-*cis*-retinol (Deigner et al., 1989). In warm-blooded mammals, at least, the 11-*cis*-retinol is oxidized to 11-*cis*-retinal in the pigment epithelium (Jones et al., 1989) and returned to the rod outer segments, where it recombines with opsin to form rhodopsin.

The retinoid isomerization process is an intriguing one because it involves the input of chemical energy to drive the thermodynamically uphill trans to cis isomerization reaction. 11-Cis retinoids are approximately 4 kcal/mol higher in energy than their all-trans isomers (Rando & Chang, 1983). This problem is solved in the visual system by a mechanism that involves the initial esterification of vitamin A, catalyzed by a lecithin retinol acyltransferase (LRAT)[1] (Barry et al., 1989; Saari & Bredberg, 1989). This enzyme transfers a saturated acyl group from the *sn*-1 position of lecithin, thereby producing a retinyl ester and a 2-acyl lysophospholipid as products (Barry et al., 1989; MacDonald & Ong, 1988; Saari & Bredberg, 1989). In a separate reaction, the retinyl ester is isomerized with hydrolysis to produce 11-*cis*-retinol and a fatty acid (Deigner et al., 1989). In this isomerase- (or more properly isomerohydrolase-) mediated reaction, the required energy is provided by the free energy of hydrolysis of the retinyl ester (Deigner et al., 1989). Thus, retinyl ester synthesis is obligate

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[1] Abbreviations: LRAT, lecithin retinol acyltransferase; RPE, retinal pigment epithelium; MS, mass spectroscopy; BSA, bovine serum albumin; BHT, butylated hydroxytoluene; THF, tetrahydrofuran; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PA, phosphatidic acid.

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in the isomerization pathway, a point explicitly demonstrated with specific inhibitors of LRAT (Trehan et al., 1990). Inhibitors of LRAT act pleitropically by preventing the conversion of *all-trans*-retinol to *all-trans*-retinyl esters and preventing the formation of 11-*cis*-retinol (Trehan et al., 1990). The inhibitors do not prevent the processing of *all-trans*-retinyl esters into 11-*cis*-retinol (Trehan et al., 1990).

Since the retinyl esters are generated with phospholipids as the source of acyl groups, the energy to drive the isomerization reaction resides in the phospholipid membranes. Hence, the phospholipids are being used as ATP surrogates in group transfer reactions in the membrane (Deigner et al., 1989).

The specificities and mechanisms of action of the LRAT and isomerase enzymes remain unexplored. In this paper, the specificities of the two enzymes with respect to substrates are studied, and aspects of the isomerase mechanism are determined. LRAT is specific for phosphatidylcholine and regiospecific for the 1-position of this phospholipid. Only retinols and similar molecules are esterified by the enzyme. Substrate/activity profiles and mechanistic studies on the isomerase demonstrate that it is highly specific and that it is extremely unlikely that it functions through a carbanion mechanism.

Materials and Methods

Materials

Frozen eye cups devoid of retinae were obtained from J. A. and W. L. Lawson Co., Lincoln, NE. [11,12-^{3}H]-*all-trans*-Retinol (60 Ci/mmol) and [7(n)-^{3}H]cholesterol (**19**) (7.8 Ci/mmol) were obtained from Du Pont-New England Nuclear, and sodium boro[^{3}H]hydride (20 Ci/mmol, 1.25 mM in 0.1 M sodium hydroxide) was from Amersham; retinal, retinol (**1**), cinnamaldehyde, cinnamyl alcohol (**16**), citral, geraniol (**17**), tetradecanal, and tetradecanol (**18**), were from Fluka; β-ionone and deuterium oxide (99.9% deuterium) were from Aldrich, and 1,2-divaleroyl-*sn*-glycero-3-phosphocholine (divaleroyl-PC) was from Sigma. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (dioleoyl-PC), 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (dioleoyl-PS), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (dioleoyl-PE), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (dioleoyl-PG), 1,2-dioleoyl-*sn*-glycero-3-phosphatidic acid (dioleoyl-PA), and 1-oleoyl-*sn*-glycero-3-phosphocholine (monooleoyl-PC) were from Avanti Polar Lipids. HPLC-grade solvents were from J. T. Baker.

Methods

Syntheses of Retinol Analogues. The retinals were prepared according to the indicated published procedures. The all-trans, 11-cis, and 13-cis isomers of the retinol analogues, unless specified otherwise, were prepared under dim red light by $NaBH_4$ reduction of the corresponding HPLC-pure retinal isomers; each alcohol was then purified by HPLC and its configuration ascertained by PMR and UV spectroscopy.

all-trans-Retinyl methyl ether (**2**) was synthesized as described earlier (Hanze et al., 1948). *all-trans*-15-Methylretinol (**3**) was synthesized as described in the published synthesis of 15-methylretinone (Ebrey et al., 1975). *all-trans*-10,12-Ethyleneretinal (**4**) was prepared according to the published method (Fang et al., 1983). *all-trans*-Phenylretinal (**5**) and *all-trans*-(2,6-dimethylphenyl)retinal (**6**) were synthesized by the published method (Derguini et al., 1984). *all-trans*-5-Demethylretinal (**7**) was obtained according to Courtin et al. (1987). *all-trans*-13-Demethylretinol (**8**) was synthesized as described earlier (Gartner et al., 1980). *all-trans*-9-Demethylretinol (**9**) was a gift of W. Gartner. *all-trans*-9,11-Trimethyleneretinol (**10**) [or 3-methyl-5,7-trimethylene-(2,6,6-trimethyl-1-cyclohexenyl)-2,4,6,8-nonatetraen-1-ol] was a gift of M. Sheves. *all-trans*-19,19,19-Trifluororetinal (**11**) was prepared according to Hanzawa et al. (1985).

all-trans-19,19,19-Trideuterioretinal (**12**) was synthesized from 9-(trideuteriomethyl)-β-ionone [*trans*-1,1,1-trideuterio-4-(6,6,2-trimethylcyclohex-1-enyl)-3-buten-2-one], which was obtained by condensation of CD_3MgI with *trans*-3-(6,6,2-trimethylcyclohex-1-enyl)-2-propen-1-al followed by MnO_2 oxidation. 9-(Trideuteriomethyl)-β-ionone was then converted to all-trans and 9-cis C_{15} aldehyde through condensation with the anion of (trimethylsilyl)acetaldehyde *tert*-butylimine. The trans C15 aldehyde isomer was converted into the desired chromophore through a conventional Emmons reaction with the anion of ethyl phosphonosenecioate (Pattenden & Weedon, 1968), Dibal reduction, MnO_2 oxidation, and HPLC separation of isomers.

all-trans-5-Methyl-7-(2,6,6-trimethyl-1-cyclohexenyl)-2,4,6-heptatrien-1-ol (C_{17} alcohol) (**13**) and *all-trans*-3-methyl-5-(2,6,6-trimethyl-1-cyclohexenyl)-2,4-pentadien-1-ol (C_{15} alcohol) (**14**) were intermediates in the synthesis of **8** (Gartner et al., 1980).

Synthesis of Radioactive Analogues. The 15-^{3}H-labeled *all-trans*-retinol analogues (**3**–**12**) were prepared by reduction of an excess of the corresponding aldehydes, dissolving in 200 μL of 2-propanol (distilled from $NaBH_4$), with 100 μL of sodium boro[^{3}H]hydride solution. The labeled alcohols were extracted with hexane and purified by HPLC on a silica column (SiO_2, 4 × 250 mm Hibar, Merck). The eluting solvent was 7% dioxane in *n*-hexane at 2 mL/min [2.5 mL/min in the case of the aromatic analogues (**5** and **6**)] or hexane–ethyl acetate–2-propanol (90:10:1) at 1 mL/min with **4**. The peak corresponding to the all-trans alcohol was collected and stored in the presence of BHT at −20 °C until use. The recovery of radioactivity in the all-trans alcohols was from 10 to 25%. The label in the 15-position of *all-trans*-retinol was not lost during its isomerization (Bernstein et al., 1987b). The other ^{3}H-labeled hydrophobic alcohols (**13**–**18**) and [15-^{3}H]-11-*cis*-retinol (**20**) were prepared and purified in a similar way.

[11,12-^{3}H]-*all-trans*-Retinyl methyl ether (**2**) was prepared as published (Hanze et al., 1948) but with radioactive retinol as starting material. [11,12-^{3}H]-*all-trans*-15-Methylretinol (**3**) (5 Ci/mmol) was obtained by reaction of methyllithium with [^{3}H]-*all-trans*-retinal at 0 °C in ether.

Preparation of Standard Isomeric Mixtures. The mixtures of each analogue were prepared by mixing the reduced isomerically pure aldehydes previously identified by HPLC. The mixture of palmitate esters was made in a similar way by acylating isomerically pure alcohols with palmitoyl chloride and pyridine in dry THF (Isler et al., 1949). Valerate esters of **1**, **13**, and **14** were prepared by the same procedure.

Enzymatic Assays. Bovine pigment epithelium membranes were prepared as described elsewhere (Barry et al., 1989) and irradiated with UV light to deplete endogenous retinols (Deigner et al., 1989). A stock suspension (1.1 μM) of each of the all-trans alcohols was prepared as follows: 5 μCi of the all-trans alcohol (5 Ci/mmol) in hexane was dried with a gentle N_2 stream. BSA (fat free), 100 μL (5% in phosphate buffer, 50 mM, pH 7.4), was added to the dried residue. The mixture was vortexed and then diluted with 800 μL of phosphate buffer. An aliquot (180 μL) of this suspension was mixed with 20 μL of pigment epithelium membranes (10 mg/mL). Final concentrations in the standard experiments were 1 mg/mL protein, 1 μM labeled substrate, and 0.5% (w/v) BSA. The mixture was incubated for 1 or 2 h at 37

°C with shaking. Control experiments were performed with buffer or boiled membranes instead of enzymatically active membranes.

Partially purified enzyme was used where indicated. In this case, the LRAT activity was solubilized and purified as previously described (Barry et al., 1989). Stock solutions of the radioactive alcohols were prepared in the same way as before. The incubations were performed with 0.07 mg of protein/mL, 1 μM labeled substrate, 1 mM divaleroyl-PC, and 0.5% BSA. Times of incubation were up to 1 h at 37 °C.

When different lipids were used, a stock suspension (3.2 mM) of each lipid was prepared by sonication of the lipid in phosphate buffer. Final lipid concentrations in the assay were 0, 0.2, 0.4, or 0.8 mM along with 0.06 mg of protein per milliliter of partially purified enzyme, 0.5 μM [15-^{3}H]-*all-trans*-retinol, and 0.5% BSA. The mixtures were incubated for 1 h at 37 °C.

The incubations were quenched by the addition of 500 μL of a cold mixture of methylene dichloride–methanol (1:1). After vortexing, 100 μL of brine was added and the mixture vortexed again. The phases were separated by centrifugation. The lower phase was removed and dried with N_2. The upper layer was reextracted with 500 μL of hexane. The hexane layer was added to the dried extract. The extent of extraction was higher than 95% as determined by measuring the radioactivity remaining in the complete aqueous phase and in an aliquot of the hexane phase.

Enzymatic Reactions Carried Out in D_2O. RPE membranes were prepared as described but with D_2O instead of water in all buffers. The buffer components were allowed to exchange with D_2O followed by evaporation of the deuterated water. This cycle was repeated several times to remove the exchangeable H. A mixture of *all-trans*-retinol (2 μM) and RPE membranes (1 mg of protein/mL) in 15 mL of deuterated buffer with 0.5% BSA was incubated for 2 h at 37 °C. Cold methanol (10 mL) was added, and the retinoids were extracted twice with hexane (10 mL). The retinoids were purified by HPLC. 11-*cis*- and 13-*cis*-Retinols were separated from *all-trans*-retinol and the endogenous cholesterol with 10% diethyl ether in hexane as the eluant. A second purification with 7% dioxane in hexane separated 11-*cis*- from 13-*cis*-retinol and *all-trans*-retinol from cholesterol. Each isomer was analyzed separately by field desorption MS as described (Deigner et al., 1989). The ratio $M + 1/M_{286}$ was calculated from the mass spectra. An experiment to control for the possible occurrence of nonspecific exchange was carried out on a heat-denatured membrane preparation in D_2O with 11-*cis*-retinol instead of *all-trans*-retinol.

Kinetic Studies. [15-^{3}H]-*all-trans*-Retinol (**1**) and [15-^{3}H]-*all-trans*-19,19,19-trideuterioretinol (**12**) were prepared with the same sodium boro[^{3}H]hydride. Assays with RPE membranes (0.5 mg/mL) and concentrations of **1** between 0.2 and 4 μM (actual concentrations of substrates were calculated by measuring the radioactivity in an aliquot of the incubation mixtures) were conducted for up to 30 min at 37 °C. Identical experiments were performed with **12**. Under the assay conditions, the isomerization was linear with respect to protein concentration.

HPLC Analyses. A 150-μL aliquot of the hexane phase, to which a standard mixture of isomeric alcohols was added for UV detection, was analyzed by HPLC under the conditions described for the purification of the ^{3}H-labeled all-trans alcohols. The percentage of isomerization and esterification was measured as the percentages of radioactivity associated with the 11-cis alcohol and esters peaks, respectively, in the chromatogram (Bernstein et al., 1987a). The radioactivity was measured with a Berthold radioactivity detector on line with the HPLC system.

Table I: Substrate Specificity for the Isomerase

analogue[a]	11-cis alcohol[b]	retinyl ester[c]	11-cis esters[d]	total 11-cis retinoids[e]
1 (+enzyme)	12.8 ± 3.3	71.0 ± 3.9	15.8 ± 3.5	24.0 ± 3.0
1 (−enzyme)	0.5 ± 0.1			0.5 ± 0.1
2 (+enzyme)	0.2 ± 0.1			0.2 ± 0.1
2 (−enzyme)	0.1 ± 0.1			0.1 ± 0.1
3 (+enzyme)	0.6 ± 0.2	39.1 ± 24	0.4 ± 0.3	0.8 ± 0.4
3 (−enzyme)	1.0 ± 0.2			1.0 ± 0.2
4 (+enzyme)	0.2 ± 0.1	60.1 ± 5.0	2.5 ± 1.6	1.8 ± 1.2
4 (−enzyme)	0.7 ± 0.1			0.7 ± 0.1
5 (+enzyme)	0.8 ± 0.1	82.4 ± 3.1	≤2	1.9 ± 0.5
5 (−enzyme)	5.7 ± 1.0			5.7 ± 1.0
6 (+enzyme)	0.2 ± 0.2	91.7 ± 1.1	1.6 ± 0.6	1.9 ± 0.7
6 (−enzyme)	0.3 ± 0.1			0.3 ± 0.1
7 (+enzyme)	6.2 ± 0.5	74.9 ± 2.4	6.4 ± 1.0	11.0 ± 0.5
7 (−enzyme)	0.7 ± 0.3			0.7 ± 0.3
8 (+enzyme)	6.2 ± 0.6	75.0 ± 1.3	1.3 ± 0.1	7.2 ± 0.5
8 (−enzyme)	1.2 ± 0.2			1.2 ± 0.2
9 (+enzyme)	0.3 ± 0.1	88.6 ± 9.9	0.8 ± 0.1	1.0 ± 0.3
9 (−enzyme)	0.9 ± 0.1			0.9 ± 0.1
10[f] (+enzyme)	1.7 ± 0.2	94.1 ± 0.4	42.9 ± 7.9	42.1 ± 8.0
10 (−enzyme)	2.8 ± 1.5			2.8 ± 1.5
11 (+enzyme)	0.5 ± 0.1	70.3 ± 1.1	≤1	0.7 ± 0.1
11 (−enzyme)	1.0 ± 0.2			1.0 ± 0.2

[a] Each analogue was incubated for 2 h with pigment epithelium membranes (+enzyme) or with buffer only (−enzyme) as described under Methods except for compounds **2** and **3**, which were incubated with frog retinal pigment epithelium membranes under the same conditions as published (Law et al., 1988). [b] Percentage of 11-cis alcohol in the total pool of retinoids. [c] Percentage of esterified compounds in the total pool of retinoids. [d] Percentage of 11-cis isomers in the ester pool. [e] Percentage of 11-cis retinoids as alcohols and esters in the total pool of retinoids. [f] The incubation time was 1 h at 37 °C.

The analysis of the 11-cis retinoids in the ester pools was performed in a similar way. To an aliquot of the reaction mixture, the corresponding mixture of nonradioactive isomeric esters was added, and analysis was performed by HPLC (SiO_2, two columns in series, 0.7% ether in hexane, 1 or 1.5 mL/min for aromatic analogues). The palmitate esters of the 11-cis isomers of **3**, **5**, **10**, and **11** were not completely resolved from the other isomers. To resolve the isomers, an aliquot of the reaction mixture was hydrolyzed with 5% KOH in methanol (5 min at 0 °C), and the resulting retinols were analyzed as described (Bernstein et al., 1987a).

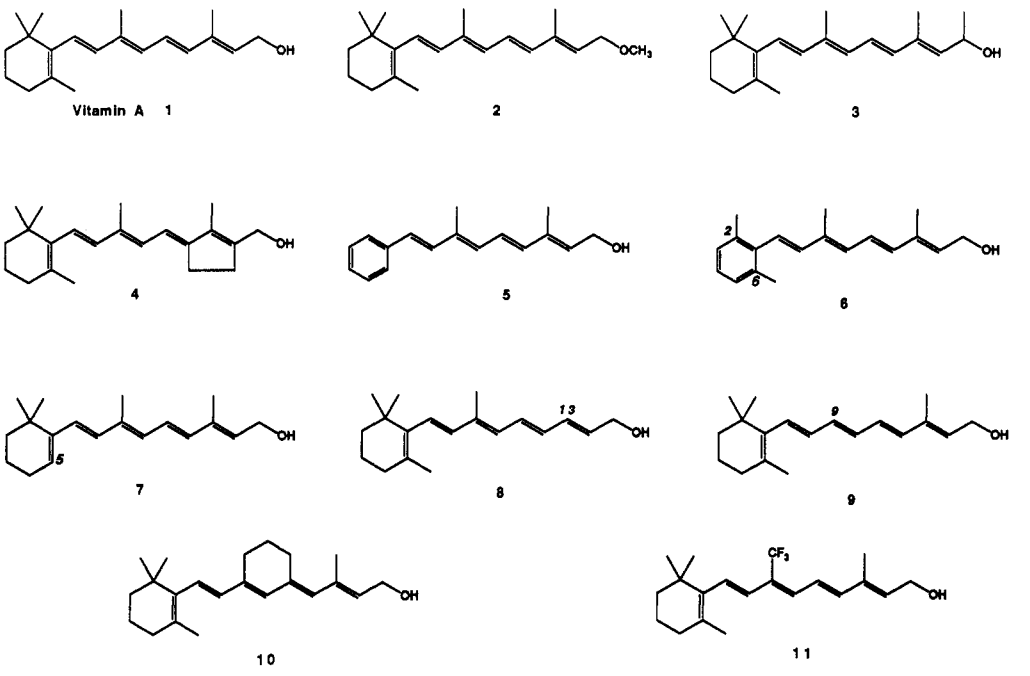
Results

Specificity of the Isomerase

We had previously shown that the isomerization reaction is stereospecific with respect to the double-bond configuration of the retinyl esters (Law et al., 1988). The isomerase only interconverts all-trans and 11-cis retinoids. Here we are interested in a broader study of the structure/activity requirements for the isomerase. It is anticipated that these studies will be important in defining the mechanism of action of this novel enzyme system. It should be noted that, in all of the cases described below where activity was observed, the retinols were first converted into their corresponding retinyl esters before isomerization occurred. The retinol analogues studied are shown in Chart I.

Modifications at the 15-Position of all-trans-Retinol. Two relatively simple molecules were initially studied (Table I). Neither *all-trans*-retinyl methyl ether (**2**) nor 15-methylretinol (**3**) was appreciably isomerized. The latter compound was esterified to its retinyl ester congener but resisted isomerization.

Chart I: Retinol Analogues



Since the former compound could not be esterified, it could not be processed into an 11-*cis*-retinol. An additional compound was studied which contained a five-membered ring adjacent to the 15-position (**4**). This molecule was again esterified but not isomerized.

Aromatic Analogues of all-trans-Retinol. Both the phenyl (**5**) and 2,6-dimethylphenyl analogues (**6**) of *all-trans*-retinol were studied (Table I). Both analogues were esterified by the endogenous LRAT activity, but neither ester was further processed to its corresponding 11-*cis*-retinol.

Modifications at the Methyl Groups of all-trans-Retinol. As one important mechanistic possibility for the isomerase involves a C–H bond abstraction followed by C–O bond cleavage, it was important to investigate the demethylretinol series (Table I). Both 5-demethylretinol (**7**) and 13-demethylretinol (**8**) were esterified by endogenous LRAT and further isomerized to provide the corresponding 11-*cis*-retinols. Interestingly, the corresponding experiment with *all-trans*-9-demethylretinol (**9**) showed esterification but no isomerization. The molecular bulk requirement at the C9 position was further studied with retinoid (**10**). This molecule was readily esterified and isomerized. In fact, it appeared to be a better substrate than *all-trans*-retinol itself. Thus, the C9 position appears not to be sensitive to steric interactions as long as there is an alkyl group present.

The possibility that C–H abstraction at the C9 methyl group of *all-trans*-retinol is important in the isomerization reaction was further considered. 19,19,19-Trifluororetinol (**11**), a molecule in which C–H abstraction is impossible, was prepared and studied (Table I). Again, esterification occurred but not isomerization. However, the strong electron-withdrawing character of the trifluoromethyl group must also be considered as a possible reason why a negative result was obtained in the isomerization reaction.

Isotope Effect and Solvent-Incorporation Studies. To further address the issue of whether C–H abstraction occurs during the isomerization reaction, the kinetic behavior of *all-trans*-19,19,19-trideuterioretinol (**11**) was studied in the isomerase reaction. Observation of a primary deuterium isotope effect would signal the occurrence of a rate-limiting C–H abstraction mechanism. We found **11** to behave almost identically with *all-trans*-retinol in its ability to be both esterified and isomerized to its 11-*cis*-retinol congener. As shown in Figure 1, the V_{max}'s for the deuterated and nondeuterated compounds were the same [approximately 0.4 nmol (mg of protein)$^{-1}$ h^{-1}] within experimental error. Furthermore, the two retinols also showed virtually identical apparent K_M's. Therefore, rate-limiting C–H abstraction at the 9-methyl does not occur during isomerization.

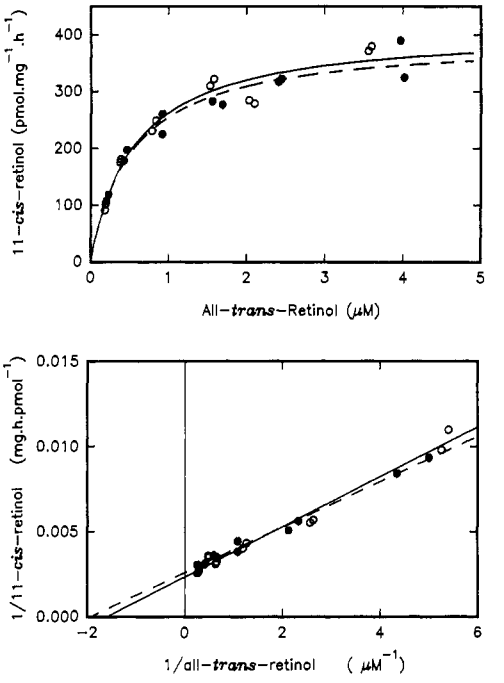


FIGURE 1: Rate of isomerization of *all-trans*-retinol (**1**) and *all-trans*-19,19,19-trideuterioretinol (**12**). Michaelis–Menten plot (upper panel) and Lineweaver–Burk plot (lower panel) of 11-*cis*-retinol production as function of *all-trans*-retinol concentration. Incubations were carried out as described under Methods. (○, —) *all-trans*-Retinol (**1**) (V_{max} = 411 ± 18 pmol mg^{-1} h^{-1}; K_m = 0.57 ± 0.5 μM); (●, – –) *all-trans*-19,19,19-trideuterioretinol (**12**) (V_{max} = 393 ± 12 pmol mg^{-1} h^{-1}; K_m = 0.53 ± 0.4 μM).

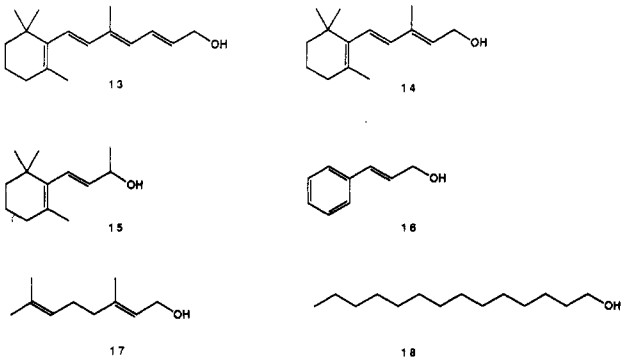
The antipode of the experiment with **11** is to carry out the isomerase experiment with *all-trans*-retinol in D_2O. Even if

Table II: Lack of Deuterium Incorporation into 11-*cis*-Retinol during Isomerization[a]

incubation of D_2O	M + 1 peak (% of M_{286} peak) of retinol isomers produced		
	11-cis	13-cis	all-trans
all-trans-retinol with enzyme	28.9 ± 3.4	27.5 ± 1.0	29.2 ± 2.6
11-*cis*-retinol with denatured enzyme	21.8 ± 4.5	24.6 ± 5.5	24.9 ± 2.9

[a] The retinol isomers obtained after incubation in D_2O were purified by HPLC and analyzed by MS as described under Methods.

Chart II: LRAT Substrates



a non-rate-limiting C–H abstraction occurred, deuterium incorporation in the product 11-*cis*-retinol would still be expected if C–H abstraction occurred. When these experiments were carried out, no significant deuterium incorporation in the 11-*cis*-retinol product was found (Table II). Taken together, these experiments render it exceedingly unlikely that a C–H bond abstraction mechanism is important in isomerase action.

Substrate Specificity of LRAT

Initial studies were aimed at determining the specificity of the enzyme for alcohol substrates. As already shown above, LRAT is broadly specific for a wide variety of *all-trans*-retinol analogues because all of the retinol analogues used to probe the isomerase [except for the methyl ether (**2**)] were esterified. It was of interest to further explore the activity of LRAT with a variety of hydrophobic alcohols as shown in Chart II. In these experiments, membrane-bound LRAT was used along with its endogenous phospholipid donor. As can be seen in Figure 2, *all-trans*-retinol and 11-*cis*-retinol were both readily esterified to form their corresponding palmitate esters. It appeared that *all-trans*-retinol might be a somewhat better substrate for the enzyme. Ionylidene analogues containing fewer carbon atoms than the retinols were studied. The C_{17} (**13**) and C_{15} (**14**) alcohols were marginally active, but β-ionol (**15**) was without activity (Figure 2). Geraniol (**17**) and tetradecanol (**18**) were both barely active as substrates, and cinnamyl alcohol (**16**) was inert (Figure 2). In separate experiments, some of the alcohols were studied with solubilized and partially purified LRAT. In these cases, divaleroyl-PC was used as the acyl donor since the endogenous lipids were removed as a consequence of the purification procedure. The results from this study (Table III) were quite similar to the earlier studies using the membrane-bound LRAT preparation. Furthermore, cholesterol (**19**) is also not a substrate for partially purified LRAT. These experiments demonstrate that LRAT is relatively specific for the retinols but can also accept other hydrophobic alcohols as substrates, albeit with substantially less efficiency than for the natural substrates.

We had previously shown that divaleroyl-PC can act as an acyl donor in LRAT (Barry et al., 1989). It was of interest to further explore the specificity of the enzyme with respect

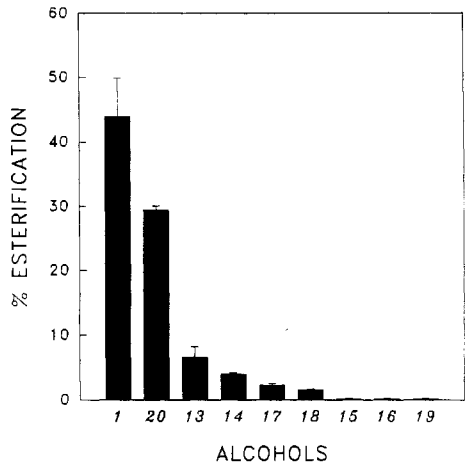


FIGURE 2: Substrate specificity of LRAT with respect to hydrophobic alcohols. In this figure **19** refers to cholesterol and **20** to 11-*cis*-retinol. Stock solutions of each alcohol were prepared as indicated under Methods and were incubated for 10 min at 37 °C with pigment epithelium membranes (0.5 mg of protein/mL). With longer incubation times or higher protein concentrations, the assay did not behave in a linear manner with respect to retinol esterification.

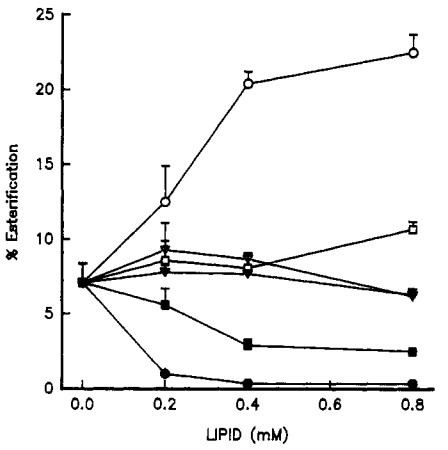


FIGURE 3: Substrate specificity of LRAT with respect to different phospholipids: (○) dioleoyl-PC; (●) monooleoyl-PC; (▽) dioleoyl-PG; (▼) dioleoyl-PS; (□) dioleoyl-PE; (■) dioleoyl-PA. Incubations were performed with partially purified enzyme as described under Methods. The percentage of esterification refers to the percentage of radioactivity in the retinyl esters pool with respect to the total amount of radioactivity added.

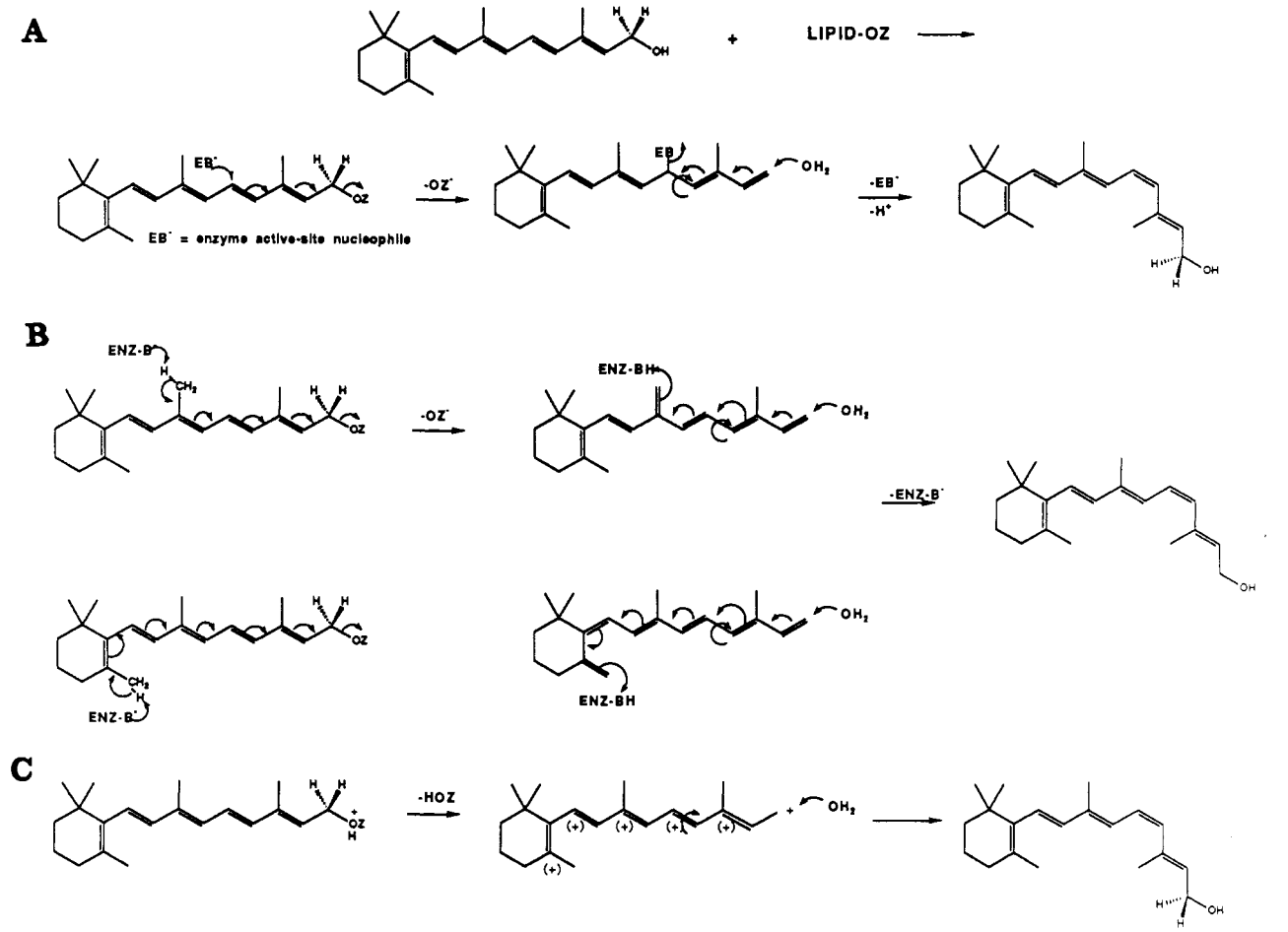
Table III: Substrate Specificity of Partially Purified LRAT with Repsect to Hydrophobic Alcohols

alcohol[a]	% esterification		% valerate ester of total esters produced
	enzyme	denatured enzyme	
1	39.5 ± 0.5	0.5	79.0 ± 0.5
13	28.6 ± 0.3	1.1	76.3 ± 2.2
14	14.7 ± 1.6	1.3	78.5 ± 0.7
19	0.8 ± 0.1	0.7	

[a] Each alcohol was incubated with partially purified enzyme as described under Methods. The esters were analyzed by HPLC with synthesized valerate esters as standards.

to acyl donors. We studied the dioleoyl derivatives of PC, PS, PE, PG, and PA. In addition, monooleoyl-PC was also studied. As shown in Figure 3, only the PC derivative proved to be substantially active as a substrate for LRAT. Of the remaining putative acyl donors, only the PE derivative showed measurable activity. Similar results have been observed with liver LRAT (MacDonald & Ong, 1988). Thus, LRAT is specific for phosphatidylcholine derivatives and is also regiospecific for the *sn*-1 position of the donor (Barry et al., 1989; MacDonald

Scheme I: Possible Isomerohydrolase Mechanisms



& Ong, 1988; Saari & Bredberg, 1989).

Discussion

The biosynthesis of 11-*cis*-retinol in the pigment epithelium of vertebrates requires two enzymatic activities (Deigner et al., 1989). First, the *all-trans*-retinol, delivered from the photoreceptors, must be esterified to produce *all-trans*-retinyl esters (Deigner et al., 1989). The *trans*-retinyl esters, chiefly of the palmitate series, are then processed directly to 11-*cis*-retinol by an isomerohydrolase enzyme (Deigner et al., 1989). Following retinol dehydrogenase mediated oxidation of 11-*cis*-retinol to 11-*cis*-retinal, the latter is transported back to the photoreceptors (at least in mammals), where it can combine with opsin to regenerate rhodopsin (Jones et al., 1989). That *all-trans*-retinyl esters are the direct precursors of 11-*cis*-retinol was unambiguously demonstrated by showing that inhibitors of LRAT, such as *all-trans*-retinyl α-bromoacetate, prevent the biosynthesis of 11-*cis*-retinol from *all-trans*-retinol (Trehan et al., 1990). The requirement of a retinyl ester precursor to 11-*cis*-retinol is understood in energetic terms (Deigner et al., 1989). In this type of reaction, the free energy of hydrolysis of the retinyl esters is coupled to the endothermic isomerization, providing the necessary free energy to drive the latter process (Deigner et al., 1989).

Several distinct mechanisms can be considered for the isomerase reaction (Scheme I). Scheme IA shows an S_N2' mechanism, Scheme IB shows C–H abstraction mechanisms, and Scheme IC shows a carbonium ion mechanism. From previous structure/activity studies it is clear that none of the dihydro analogues of *all-trans*-retinol are isomerized, although all of them are esterified by LRAT (Law et al., 1988). These observations suggest substantial substrate specificity for the isomerase, but they are not informative as to mechanism. Some of the most interesting retinoid analogues studied here focused on the role of the appended alkyl groups of *all-trans*-retinol. Both the 5- and 13-demethylretinyl esters were isomerized. These observations rule out possible C–H abstraction at either of these sites in the isomerization reaction. Previous studies (Law et al., 1988) showed that *all-trans*-3,4-didehydroretinol (vitamin A_2) was isomerized, also ruling out possible C–H abstraction from the ionylidene ring as being mechanistically important. The fact that *all-trans*-9-demethylretinyl ester was not isomerized points to a possible important role for the 9-methyl group in the isomerization process. However, this role is unlikely to involve C–H abstraction because the V_{max}'s and apparent K_M's for the isomerizations of *all-trans*-19,19,19-trideuterioretinol and *all-trans*-retinol were essentially identical. The V_{max}'s for *all-trans*-19,19,19-trideuteroretinol and *all-trans*-retinol were both approximately 0.4 nmol mg^{-1} h^{-1}. It should be noted that the absolute value for the V_{max} underestimates the true V_{max} of the isomerizing system because the amount of 11-*cis*-retinyl esters formed is not considered and the 11-*cis*-retinol that is formed is unstable and can nonenzymatically isomerize back to its all-trans congener (Deigner et al., 1989). The actual maximal rate of overall isomerization capability in the bovine pigment epithelium membranes is closer to 1–2 nmol mg^{-1} h^{-1} after corrections for the formation of 11-*cis*-retinyl esters and the thermal instability of 11-*cis*-retinol are made.

In addition to the lack of an observable kinetic isotope effect, deuterium incorporation into 11-*cis*-retinol was not observed when the isomerization reaction was performed in D_2O. The observed lack of deuterium incorporation is also inconsistent with a C–H abstraction mechanism unless the proton ab-

stracted was also added back. This could occur in the absence of H_2O, but this is considered unlikely here because water is required to hydrate the isomerization intermediate (Scheme I).

Other substituted analogues at the 9-position were also investigated. Analogue **10** was readily processed into its 11-*cis*-retinol analogue, demonstrating that sterically bulkier groups than the methyl group are allowed at the C9 position. This analogue is also interesting since its 11-cis congener is relatively stable compared to the all-trans form because of the trimethylene moiety adducted to the C11 position. In this configuration, steric interaction between this methylene moiety and the 13-methyl group renders the trans configuration unstable. This interaction is relieved in the 11-cis configuration. The fact that this analogue is active also shows that substitution at the 11-position is permitted. Given these observations, the fact that *all-trans*-19,19,19-trifluororetinol is inactive can probably be understood in electronic terms. On the basis of these studies in aggregate, it is highly unlikely that a C–H abstraction mechanism (Scheme IB) is important in the isomerase-mediated reaction.

It is interesting to compare some of these results with those obtained on analogue studies with rhodopsin. Both 11-*cis*-5- and 13-demethylretinals form biochemically competent rhodopsins (Blatz et al., 1988; Kropf, 1976). 11-*cis*-9-Demethylretinal also forms a photoisomerizable pigment with opsin (Eyring et al., 1980). However, unlike the other demethyl analogues, the pigment formed from 11-*cis*-9-demethylretinal fails to produce the biochemically active form of rhodopsin (metarhodopsin 2) upon photoisomerization (Ganter et al., 1989). It is possible that the 9-methyl group is pivotal in pushing against both the isomerases and opsin proteins concomitant with isomerization. This interaction could then lead to the key conformational changes of the proteins required to produce a fruitful biochemical event.

Consideration of the structure/activity relationships for isomerization reveals fairly strict structural requirements. The ionylidene ring system cannot be substituted for by phenyl groups (**5** and **6**), and sterically bulky groups at the C15 terminus of the retinoid are not tolerated (**3** and **4**). As expected, O-methylation of *all-trans*-retinol leads to an inert compound. Only at the center of the molecule, i.e., positions 9 and 11, is bulkier substitution allowable. As mentioned before, a substituent at the C9 position is critical for activity.

In addition to determining the specificity of an enzyme, structure/activity studies can also be important in gaining insights into enzyme mechanism. The structure/activity studies performed so far, along with the deuterium incorporation and isotope studies, are inconsistent with a C–H abstraction mechanism, and one can probably rule this mechanism out from further consideration. The studies reported here do not differentiate between the S_N2' and carbonium ion mechanisms described in panels A and C of Scheme I, respectively. The marked effect of the trifluoromethyl substitution (**11**) could suggest the possible development of carbonium ion character during isomerization. However, more complete studies with variously fluorinated retinoids will be important before further conclusions can be drawn.

All of the *all-trans*-retinol analogues studied here (except for the methyl ether) and the dihydroretinols as previously studied (Law et al., 1988) were esterified by LRAT. These observations demonstrate a relatively nonspecific enzymatic activity within the retinol series. However, LRAT is relatively specific for the retinols when compared to other hydrophobic alcohols. Truncated versions of retinols (**13** and **14**) are weak substrates, and hydrophobic alcohols structurally dissimilar to the retinols, such as cholesterol, were not esterified. As expected, 11-*cis*-retinol was also esterified, although less efficiently than *all-trans*-retinol.

The specificity of LRAT with respect to lipid donors was also studied. It is clear that, of the major lipid classes, only phosphatidylcholine analogues are substantially processed. As previously noted, acyl transfer occurs in a regiospecific manner from the *sn*-1 position of phosphatidylcholine (Barry et al., 1989; MacDonald & Ong, 1988; Saari & Bredberg, 1989). We have found little specificity with respect to the fatty acyl groups. Divaleroyl-PC is a competent acyl donor (Barry et al., 1989). However, other studies have suggested that there may be some specificity directed toward naturally occurring phospholipids with regard to the nature of the unsaturated fatty acyl group at the *sn*-2 position (Saari & Bredberg, 1989).

Since LRAT has not yet been purified to homogeneity, it is not clear if isoenzymes exist whose activities can be separately regulated. This would be especially interesting with respect to the processing of *all-trans*-retinol and 11-*cis*-retinol. *all-trans*-Retinol serves no direct metabolic function and must be esterified to produce the isomerase substrate. 11-*cis*-Retinol, on the other hand, serves as the immediate precursor for the visual chromophore 11-*cis*-retinal. The esterification of 11-*cis*-retinol serves no obvious metabolic purpose other than to provide a storage mechanism. It is interesting that steady-state levels of 11-*cis*-retinol are much higher than those of *all-trans*-retinol in the dark (Hubbard & Dowling, 1962).

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all-trans-19,19,19-Trifluororetinol and *all-trans*-9-demethylretinol were generous gifts of Dr. Y. Kobayashi of the Tokyo College of Pharmacy and Dr. W. Gartner of the University of Freiburg. The cyclic *all-trans*-retinol (**10**) was a generous gift of Dr. M. Sheves of the Weizmann Institute of Science. Dr. Achla Trehan synthesized *all-trans*-13-demethylretinol and compounds **13** and **14**. We are indebted to Dr. T. Higuchi of JEOL Inc. for the mass spectroscopic measurements. The excellent technical assistance of M. Sarma is gratefully acknowledged.

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Fusion of Influenza Hemagglutinin-Expressing Fibroblasts with Glycophorin-Bearing Liposomes: Role of Hemagglutinin Surface Density†

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ABSTRACT: Influenza virus gains access to the cytoplasm of its host cell by means of a fusion event between viral and host cell membrane. Fusion is mediated by the envelope glycoprotein hemagglutinin (HA) and is triggered by low pH. To learn how many hemagglutinin trimers are necessary to cause membrane fusion, we have used two NIH 3T3 fibroblast cell lines that express HA protein at different surface densities. On the basis of quantitations of the number of HA trimers per cell and the relative surface areas of the two cell lines, the HAb-2 cells have a 1.9-fold higher plasma membrane surface density than the GP4F cells. The membrane lateral diffusion coefficient and the mobile fraction for HA is the same for both cell lines. A Scatchard analysis of the binding of glycophorin-bearing liposomes to the cells showed 1700 binding sites for the GP4F cells and 3750 binding sites for the HAb-2 cells, with effectively the same liposome–cell binding constant, about 7×10^{10} M^{-1}. Binding was specific for glycophorin on the liposomes and HA expressed on the cells. A competition experiment employing toxin-containing and empty liposomes allowed us to quantitate the number of liposomes that fused per cell, which was a small constant fraction of the number of bound liposomes. For the HAb-2 cells, about 1 in every 70 bound liposomes fused and for the GP4F cells about 1 in every 300 bound liposomes fused. Hence, the HAb-2 cells showed 4.4 times more fusion per bound liposome, even though the surface density of HA was only 1.9 times greater. We conclude the following: (i) One HA trimer is not sufficient to induce fusion. (ii) The HA bound to glycophorin is not the HA that induces fusion. That is, even though each HA has a binding and a fusion function, those functions are not performed by the same HA trimer.

Hemagglutinin, the major envelope glycoprotein of influenza virus, is responsible for binding the virus to sialic acid containing receptors on the host cell surface (Hirst, 1941) and for initiating the fusion event between viral and cellular membranes [e.g., Maeda and Ohnishi (1980), Matlin et al. (1981), and White et al. (1982)]. The ectodomain of HA[1] has been crystallized (Wilson et al., 1981) and shown to undergo a conformational change at the pH associated with viral fusion (Skehel et al., 1982; Doms et al., 1985; Ruigrok et al.,

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[1] Abbreviations: HA, hemagglutinin; HAO, the fusion-incompetent hemagglutinin precursor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PBS CMF, Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PMSF, phenylmethanesulfonyl fluoride; RBC, red blood cell; RIA, radioimmunoassay; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

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