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Studies on the transacylation of retinol catalyzed by acyl coenzyme A:retinol O-acyltransferase

Mark David Ball
Iowa State University

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Studies on the transacylation of retinol catalyzed by acyl coenzyme A:retinol O-acyltransferase

Ball, Mark David, Ph.D.

Iowa State University, 1987
Studies on the transacylation of retinol catalyzed
by acyl coenzyme A:retinol O-acyltransferase

by

Mark David Ball

A Dissertation Submitted to the
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For the Major Department
Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa
1987
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ABBREVIATIONS USED

ACAT  acyl CoA:cholesterol O-acyltransferase
all-t-RA  all-trans-retinoic acid
ARAT  acyl CoA:retinol O-acyltransferase
BP  benzo(a)pyrene
BSA  bovine serum albumin
CHAPS  3-[(3-cholamidopropyl)-dimethylammonio]-l-propane sulfonic acid
CRPB(II)  cellular retinol-binding protein, type "II"
DMSO  dimethylsulfoxide
DTT  dithiothreitol
EDTA  ethylenediaminetetraacetic acid
HPLC  high-pressure liquid chromatography
HPR  N-(4-hydroxyphenyl)retinamide
$k_{cat}$  ratio of $V_{max}$ to enzyme concentration
$K_m$  substrate concentration at 50% $V_{max}$
MeROL  15-methylretinol
M6P  mannose-6-phosphate
M6Pase  mannose-6-phosphatase
NADPH  reduced nicotinamide adenine dinucleotide phosphate
RBP  retinol-binding protein
REH  retinyl ester hydrolase
TCDD  2,3,7,8-tetrachlorodibenzo-p-dioxin
13-c-RA  13-cis-retinoic acid
\[ v \]

\[ v \quad \text{velocity} \]

\[ v_{\text{max}} \quad \text{maximal velocity} \]
GENERAL INTRODUCTION

Acyl coenzyme A:retinol O—acyltransferase (ARAT; EC 2.3.1.76) is a microsomal enzyme that catalyzes the esterification of retinol with fatty acids, using a fatty-acyl-CoA thioester as donor of the acyl group (1). Inasmuch as vitamin A is stored in the liver as esters of retinol (2), ARAT may participate directly in the generation of an animal's reserves of this vitamin in vivo. Because the present work is a study of this transacylation and of certain biochemical properties of ARAT, and because the results are interpreted physiologically, both the biochemistry of vitamin A and the enzymology of ARAT are reviewed here.

Biochemistry of Vitamin A

Unable to synthesize vitamin A, animals must rely on their diet to provide it. The ultimate chemical source of vitamin A for animals is the group of fat-soluble plant pigments known as "carotenoids". Although several hundred carotenoids are known (3), only about 50 have the potential to serve as precursors of vitamin A in the animal (4). Of these vitamin-A-active carotenoids, β-carotene is the most potent as well as the most abundant (5).
After being eaten by the animal, β-carotene enters the lumen of the small intestine, where, because of its hydrophobicity, it is emulsified with other dietary lipids by bile salts. The micelles so formed passively diffuse across the microvillus membrane and into the mucosal cell (6). The β-carotene is then cleaved at the 15-15' double bond by a dioxygenase in the mucosal cell to yield two molecules of retinal (7,8). Evidence for a second mechanism whereby β-carotene is cleaved has been reviewed by Ganguly and Sastry (9). Cleavage in this mechanism is random rather than central, yielding only one molecule of retinal. Irrespective of mechanism, newly generated retinal is then reduced to retinol by a thiol- and pyridine-nucleotide-dependent aldehyde reductase (10). In the gut, retinol also arises directly from the hydrolysis of retinyl esters (11), the form of vitamin A in foods of animal origin, in fortified foods (e.g., milk and breakfast cereals), and in vitamin pills.

A cellular retinol-binding protein, type "II", (CRBP (II)), then binds its insoluble ligand, retinol, carrying it to intracellular sites of esterification (12). Although retinol is esterified in the small-intestinal mucosa (13,14), the role of ARAT in catalysis of the reaction in vivo is uncertain (14), especially with the recent discovery of an acyl-CoA-independent retinol acylase in the small intestine (15). The relative contribution of these enzymes to the total
rate of retinol esterification in vivo is unknown. Regardless of the enzyme, newly formed retinyl esters are subsequently packaged into the hydrophobic core of chylomicrons, along with other dietary lipids, including absorbed β-carotene. Secreted by the enterocytes into surrounding lymph, chylomicrons ultimately enter the blood through the thoracic duct (16).

As the chylomicrons circulate, the core triacylglycerols are hydrolyzed by lipoprotein lipase on the surface of capillary endothelium (17), whereas the retinyl esters escape hydrolysis during this phase of chylomicron processing (18). Thereby enriched in vitamin A, the "chylomicron remnants" arising from this lipolysis are eventually removed from circulation by the liver (19) in a receptor-mediated endocytosis. After fusion of lysosomes with the vesicles created by this endocytosis, degradation of the vesicles' contents begins (20). There is evidence, however, that vitamin A in the endocytosed vesicles is transferred directly to the endoplasmic reticulum rather than being engulfed by lysosomes (21).

Although it has been known for many years that retinyl esters in the chylomicron remnant are hydrolyzed after reaching the liver (22), it is not known whether hydrolysis occurs before fusion of the endocytosis vesicles with lysosomes, catalyzed presumably by a plasma-membrane-bound hydrolase, or after fusion, catalyzed by a lysosomal acid
hydrolase. After hydrolysis of its esters, hepatic retinol will follow one of three possible paths: 1) binding to plasma retinol-binding protein (RBP) for secretion into the blood, 2) oxidation or conjugation for excretion by the animal, and 3) esterification and storage in the liver.

The binding of retinol to apo-RBP and the subsequent secretion of holo-RBP are particularly rapid when hepatic reserves of vitamin A are low (23,24). Retinol is bound by apo-RBP in the endoplasmic reticulum, and the holo-RBP so formed is then secreted into the blood in Golgi-derived vesicles (25,26). In retinol deficiency, apo-RBP accumulates in the liver (27,28), but immediately binds retinol upon repletion, quickly entering the blood as holo-RBP (28).

Reactions whereby retinoids are oxidized or conjugated in cell-free systems or by cells in culture are numerous. Retinol can be oxidized reversibly to retinal by a pyridine-nucleotide-dependent enzyme present in the small intestine (29), liver (30), and eye (31). Retinal, in turn, can be oxidized to retinoic acid by another pyridine-nucleotide-dependent enzyme that has been identified in several tissues, including the liver (32,33). Whereas the dehydrogenase for retinol and for ethanol may be the same enzyme in the liver (30), in kidney cells they are distinct (34).

The interconversion of retinol and retinal is important physiologically in at least two respects: 1) retinol must be
oxidized to retinal in the eye because the-aldehyde is the prosthetic group of rhodopsin (35), and 2) retinal must be reduced to retinol in the small intestine after oxidative cleavage of carotenoids in order for these pigments ultimately to have provitamin A activity.

On the contrary, the importance of the irreversible oxidation of retinal to retinoic acid is physiologically obscure. Although the acid is generally more active than other retinoids in cell-culture systems (36), whether or not retinoic acid is an obligatory metabolite of vitamin A is unknown. Among the many enzyme-catalyzed reactions that retinoic acid itself can undergo in vitro are glucuronidation (37), decarboxylation (38), epoxidation (39), and isomerization (40).

Unfortunately, most retinoid metabolites occurring in urine, bile, and feces have not been characterized well. Water-soluble metabolites of retinol, presumably oxidation products, have been found in the urine (22). Retinyl β-glucuronide has been identified in bile (41), along with retinoic acid and its conjugates (42). However, a recent study unequivocally identified 4-hydroxyretinol among the polar metabolites of retinol in incubations with rat-liver microsomes (43). The formation of 4-hydroxyretinol was dependent upon O₂, NADPH, and cytochrome P-450. Because no retinoic acid was detected among the reaction products, the
authors concluded that retinoic acid is not an obligatory intermediate in the pathway for retinol oxidation catalyzed by liver microsomes. Furthermore, $V_{\text{max}}$ and $K_m$ are such that this pathway may be physiologically significant.

Mammalian microsomes also catalyze the transfer of mannose from GDP-mannose to retinyl phosphate (44,45). Retinol can be phosphorylated \textit{in vitro} (46-49) in an ATP-dependent reaction (46), and, indeed, $\beta$-mannosylphosphorylretinol (retinyl phosphate mannose) has been isolated from whole liver (49) and from hepatic membrane preparations (50). Although only a small proportion of hepatic vitamin A is in the form of retinyl phosphate mannose at any given time (49), the theory that vitamin A functions in the mannosyl-transfer reactions of glycoprotein biosynthesis has been proposed (51).

Another microsome-catalyzed reaction generates retinyl $\beta$-glucuronide (41), already mentioned as a metabolite in bile. In this non-oxidative conjugation, uridine diphosphoglucuronic acid donates the glucuronyl moiety to retinol to form the acetal product. Although the physiological role of this compound is still a mystery, its presence in human blood has been proven (52).

The most thoroughly studied reaction of retinol, however, is also the one whose physiological role in the biochemistry of vitamin A is relatively well-understood. This reaction is esterification, catalyzed by ARAT, mentioned above.
Acyl Coenzyme A:Retinol O-Acyltransferase

ARAT is a microsomal enzyme that catalyzes the transacylation of retinol with fatty-acyl-CoA thioester derivatives (1). Present in a large variety of tissues and species, this enzyme has been identified in the liver of the rat (1), human (53), and chick (54), as well as in the mucosa of rat (55) and human (56) small intestine. ARAT activity has also been seen in lamb kidney (57), in a cell line derived from pig kidney (34), and in rat mamma (58), rat testis (59), and mouse epidermis (60). Uncharacterized retinol-acylating activity has been detected in rabbit lung (61) and in retinal pigment epithelium from the frog (62), human (63), and cow (64).

The in-vitro esterification of retinol by a cell-free system was first reported by Futterman and Andrews in 1964 (2). Using cat liver, these workers found that most of the retinol-acylating activity was associated with the microsomes. Finding no requirement for supplements or cofactors (e.g., ATP and CoA), they concluded that the acylation reaction was energy-independent. In a 1982 report by Ross (1), however, the enzyme was characterized, but the data contradicted the conclusion of Futterman and Andrews (2).

Ross (1) showed stimulation of the in-vitro esterification of retinol by addition to the reaction mixture of either a...
fatty-acyl-CoA thioester derivative or a fatty acid with CoA and ATP. Furthermore, when \[^3H\]retinol and \[^{14}C\]palmitoyl CoA were incubated with microsomes, the ratio of \(^3\)H to \(^{14}\)C in the ester product was the same as in the substrates, evidence that the palmitoyl moiety was transferred directly to retinol, rather than first to an intermediate. Thus, Ross (1) classified the retinol-acylating activity as an acyltransferase.

Ross (1) observed esterification even in the absence of exogenous fatty-acyl CoA, as long as retinol was included in the incubation with microsomes. Additional, albeit indirect, support for classifying the enzyme as an acyltransferase arose from elimination of this basal rate by treatment of the microsomes with hydroxylamine before incubation with retinol. Inasmuch as hydroxylamine reacts with fatty-acyl-CoA thioesters to form hydroxamates (65), fatty-acyl CoA must have been endogenous to the microsomes used by Ross. Because other reports for various tissues and species also have shown a requirement for activated fatty acids (52-60), the energy dependence of at least one microsomal retinol-acylating enzyme is now clear.

Although the chemical mechanism of the transacylation catalyzed by ARAT has not been probed, sulfhydryl groups are known to be necessary for activity, whereas serine hydroxyl groups are probably unimportant. ARAT is inhibited by p-
chloromercuribenzoate (1,58), p-hydroxymercuribenzoate (59,60) 5,5′-dithiobis-(2-nitrobenzoic acid) (55,56,59), and p-chloromercuriphenylsulfonate (59), all of which are sulfhydryl-blocking agents, but not by phenylmethylsulfonyl fluoride (58,60), an inhibitor of serine-dependent enzymes. A requirement for metal ions is also unlikely inasmuch as EDTA fails to inhibit ARAT in vitro (58,59). Exogenous thiols, including dithiothreitol (1,58-60), glutathione (1), and cysteine (1), as well as bovine serum albumin (1,55,56,58,59), accelerate the reaction in vitro when included in the incubation mixtures, although most reports are cursory on this point.

Whereas the chemical events of the transacylation have not been elucidated, the physiological significance of ARAT has been investigated. ARAT is not the same microsomal enzyme that catalyzes the analogous transacylation of cholesterol, acyl CoA:cholesterol O-acyltransferase (ACAT; EC 2.3.1.26). Their being the same enzyme has been precluded by at least five observations: 1) that retinol esterification was not inhibited in vitro, competitively or otherwise, by cholesterol (1), 2) that cholesterol esterification was inhibited in vitro by (3-[decyldimethylsilyl]-N-[2-(4-methylphenyl)-1-phenylethyl]propanamide), whereas retinol esterification was not (66), 3) that, in the absence of added fatty-acyl CoA, cholesterol is esterified in vitro with mainly unsaturated
fatty acids (67), whereas those with which retinol is esterified are primarily saturated (2), 4) that treatment of cultured retinal pigment epithelial cells with butyrate stimulates the intracellular esterification of retinol but not that of cholesterol (63), and 5) that treatment of cultured rat–liver cells with 25-hydroxycholesterol or mevalonolactone accelerates the intracellular esterification of cholesterol but not that of retinol (68). The in-vitro distinction between ARAT and ACAT satisfies the expectation that vitamin A and cholesterol, two compounds with very different physiological roles, are routed into mutually exclusive biochemical pathways.

In contrast to β-carotene (69), vitamin A is potentially toxic (70). Moore speculates in his treatise on vitamin A that, inasmuch as the liver continues to function normally even when its concentration of vitamin A is abnormally high, esterification may be the liver’s way of detoxifying retinol (71). Although not lowered by vitamin–A deficiency (72), ARAT activity is enhanced in liver microsomes from both rats (72) and chicks (73) on high vitamin–A intakes, but is not altered by the administration of retinoic acid (72). Consequently, if ARAT is present in species with exceptionally large hepatic vitamin–A reserves, e.g., the polar bear (74,75), its activity is expected to be higher in vitro than that of ARAT from most other species.
Vitamin-A toxicosis probably arises only when absorption of the vitamin by the intestine exceeds the liver's capacity for removing vitamin A from the blood, for esterifying it, and for sequestering it as it does, in vitamin-A-rich globules (76). Thus, ARAT's presence in the liver is a two-fold advantage in the sense that it 1) generates the chemical storage form of vitamin A, and 2) detoxifies retinol. Moore notes the irony that "...animals with high reserves of vitamin A might be in danger of poisoning themselves if they were able to eat their own livers" (71).

While hepatic ARAT is inhibited in vitro by progesterone, deoxycholate, and taurocholate (1), retinol acylation by microsomes from various other tissues (whether or not the activity has been identified as ARAT) is inhibited by Triton X-100 (58), bile-salt detergents (55,56,58,59), and by retinoids, including 3,4-didehydroretinol (60,62), all-trans-retinoic acid (60,77), 13-cis-retinol (60) and 4-hydroxyretinol (60). Strictly speaking, 3,4-didehydroretinol, 13-cis-retinol, and 4-hydroxyretinol probably do not truly inhibit the enzyme; they are more properly regarded as competing substrates, and, at least in the case of 3,4-didehydroretinol (62), inhibit by virtue of being esterified themselves. Whereas the in-vitro inhibition of ARAT by detergents suggests that this membrane-bound enzyme is sensitive to changes in its lipid environment, the inhibition
of small-intestinal ARAT by all-trans-retinoic acid (77) was interpreted as a threat to the synthesis and absorption of retinyl esters in cases where retinoic acid or similar retinoids are ingested in large quantities, e.g., therapy for acne. The inhibition of epidermal ARAT by retinol analogs (60) was interpreted non-physiologically by the authors as the absence of absolute enzyme specificity for the alcohol substrate.

The predominant vitamin-A ester in the liver is retinyl palmitate, with smaller amounts of stearate, oleate, and linoleate (2,22,78). Futterman and Andrews (2) reported that, with no added fatty-acyl CoA, microsomes esterified retinol with a variety of fatty acids, namely, palmitate, stearate, palmitoleate, oleate, and linoleate. Although palmitoyl CoA has been the substrate of choice for in-vitro studies on ARAT in various tissues (1,53,55,56,58-60,72,77), the stearoyl (1,56,58,59), oleoyl (1,55,56,58,59), linoleoyl (60), lauroyl (1,58), and octanoyl (1,58) thioesters of CoA have been tested as substrates for the enzyme.

For ARAT from rat (55) and human (56) small intestine, stearoyl and oleoyl CoA were less effective as substrates than palmitoyl CoA. The relationship was different, however, for lactating rat mamma: oleoyl > stearoyl > palmitoyl > lauroyl > octanoyl CoA (58). For rat testis, palmitoyl, stearoyl, and oleoyl CoA were equally effective substrates (59). In assays
for ARAT from mouse epidermis, linoleoyl CoA "could replace palmitoyl CoA as acyl donor", but their relative rates are not given in the report (60). Finally, hepatic ARAT showed still another order of substrate-effectiveness: palmitoyl > lauroyl > oleoyl > stearoyl > octanoyl CoA (1).

No major research focusing on the enzymology of retinol esterification emerged between the report in which Futterman and Andrews demonstrated the in-vitro esterification of retinol by cat-liver microsomes (2) and the report by Ross in which the activity was characterized (1), an interim of 18 years. Despite the ensuing flurry of research on ARAT, surprisingly little information has accrued on three particular fundamental biochemical and physiological aspects of the enzyme and the reaction it catalyzes. The research presented herein addresses these three issues.

Firstly, if ARAT is indeed physiologically responsible for generating the chemical storage form of vitamin A, is the enzyme present in the liver of species whose reserves of vitamin A are naturally very large? If so, how do its activity and characteristics compare with those for other species? Furthermore, is ARAT present in tissues that do not accumulate retinyl esters, and, if so, can its activity be enhanced in these tissues by administration of vitamin A to the animal?
Secondly, although ARAT from other tissues is inhibited in vitro by various retinoids (60,62,77), both natural and synthetic, the specificity of the inhibition (i.e., whether or not these compounds affect other microsomal enzymes) has not been studied. Furthermore, is hepatic ARAT subject to inhibition by retinoids, and, if so, how inhibitory are those used therapeutically in the treatment of skin disorders or cancer?

Thirdly, the predominance of retinyl palmitate in the liver over esters of retinol with other fatty acids (2,22,78) implies a specificity in ARAT for palmitoyl CoA. Although various fatty-acyl-CoA thioester derivatives have been compared as substrates for the in-vitro reaction, they have been incubated with microsomes singly, rather than together as competing substrates. Furthermore, the potential complication of microsomal acyl CoA hydrolases and BSA, which binds oleoyl CoA in vitro (79), have been ignored. Reports also have overlooked polyunsaturated and odd-chain fatty-acyl-CoA thioesters as substrates for ARAT. With these considerations, then, what is the specificity of ARAT in vitro, and does it account for the fatty-acid composition of retinyl esters in vivo?
PART I.

ACYL COENZYMЕ A:RETINOL O-ACYLTRANSFERASE
IN POLAR-BEAR LIVER AND RAT MAMMARY TUMOR AND ENHANCEMENT OF
ITS ACTIVITY BY HIGH DOSES OF VITAMIN A
INTRODUCTION

Whether the animals are dosed or vitamin A is added to the diet, large increases in vitamin-A intake are accompanied by an increase in the in-vitro activity of acyl CoA:retinol O-acyltransferase (ARAT) in rat (72) and chick (73) liver, as well as in rat small intestine (72). This observation strengthens the hypothesis that ARAT is physiologically responsible, at least in part, for generating the chemical storage form of vitamin A. Justification for assigning physiological importance to ARAT also would be found in two other observations: 1) the presence of ARAT, and with an unusually high activity, in the liver of species whose reserves of vitamin A are naturally very large, and 2) the absence of ARAT, or its presence at an unusually low activity, in a tissue that does not store esters of retinol, and the enhancement of ARAT activity in such a tissue by administration of vitamin A to the animal.

Among the animals known to have exceptionally large hepatic vitamin-A stores are the polar bear (74,75), certain seals (74,80), and other marine mammals, like the bowhead whale (75). Vitamin A has been quantified in several specimens of polar-bear liver, ranging in concentration from 3900 µg/g tissue to 10,380 µg/g (74,75,81,82; concentrations
expressed as "IU/g" have been converted to "µg retinol/g" by the equation "1 IU = 0.3 µg retinol").

The concentration of vitamin A in most animal livers is normally much smaller than in that of the polar bear. For the vitamin-A-normal rat, for example, values of 43 µg/g liver (76) and 172 µg/g (72) have been reported. Typical values for man are 173 µg/g liver (83), 126 µg/g (84), and 211 µg/g (85). Also lower than that of the polar bear, the hepatic concentrations of vitamin A have been measured for many other species as well. One study involved the livers of calf, sheep, rabbit, rat, frog, rainbow trout, and the domestic cat (2), while other studies have focused on larger cats (86) and on chicks (73).

From the accidental death of a polar bear at a nearby zoo arose the rare opportunity to ascertain not only whether polar-bear liver contains an unusually active ARAT, but also 1) whether some properties of the enzyme are similar to those for ARAT from other species, 2) whether a polar bear raised in captivity also has very large hepatic reserves of vitamin A, and 3) whether the fatty-acid composition of retinyl esters in polar-bear liver is similar to that found in other species.

At the opposite extreme, examples of tissues that do not store vitamin A are rat hepatoma (87) and rat mammary tumor (88; O. Amedee-Manesme and H. C. Furr, Dept. of Biochemistry and Biophysics, Iowa State University, Ames, Iowa, personal
communication). ARAT in microsomes from another rat hepatoma cell-line (Fu5AH) catalyzed retinol esterification *in vitro* at a rate an order of magnitude lower than that for normal rat liver (66). Because it has not, however, been identified in a rat mammary tumor, ARAT in this tissue was assayed, and the response of its activity to administration of vitamin A to the animals was determined. For comparison, ARAT activity in the liver also was measured before and after dosing.
EXPERIMENTAL PROCEDURE

Materials

Retinal, retinyl acetate, retinyl palmitate, palmitoyl CoA, DL-dithiothreitol, bovine serum albumin (BSA; essentially globulin- and fatty-acid-free), were purchased from Sigma Chemical Co. (St. Louis, MO). n-Decane and 5-nonanone were supplied by Aldrich Chemical Co. (Milwaukee, WI). All other chemicals and solvents were of the highest quality commercially available. Under gold fluorescent laboratory lights (Sylvania F40G0) all-trans-retinol was prepared by reduction of all-trans-retinal in methanol with excess sodium borohydride, and then purified by high-pressure liquid chromatography (HPLC) on a Whatman Partisil M9 10/50 straight-phase column (9.4 mm x 50 cm), with an eluting solvent of hexane/ethyl acetate (90:10, v/v), flowing at 7 mL/min. For the final stock solution, retinol was dissolved in dimethylsulfoxide (DMSO) and its concentration was determined by absorbance in ethanol at 325 nm, with $\varepsilon = 52,480 \text{ M}^{-1} \text{ cm}^{-1}$. Palmitoyl CoA was dissolved in 150 mM potassium phosphate buffer, pH 7.4, and its concentration was determined by absorbance at 259.5 nm (at pH 7.0), with $\varepsilon = 15,400 \text{ M}^{-1} \text{ cm}^{-1}$. 
Animals

Having been raised from a cub at Brookfield Zoo in Brookfield, IL, a male polar bear (*Ursus maritimus*) suffered a broken neck as the result of a fall. Killed shortly thereafter by lethal injection, at about 10 a.m., the bear was transported to the University of Illinois at Urbana-Champaign where, at about 3 p.m., the liver was removed and frozen. At death the bear was 14 years old and weighed about 850 pounds.

While in captivity at Brookfield Zoo, the bear was maintained on Zu/Preem Omnivore Diet and Nebraska Brand canine diet (Animal Spectrum, Lincoln, NE) and Purina Field and Farm dry dog food (Ralston-Purina, Richmond, IN), raw unpeeled apples, white enriched bread, and smelt from Lake Superior. The bear's estimated daily consumption of about 13 kg of food includes 77,700 IU (23,300 µg) of vitamin A, 58% being supplied by the canine diet, 30% by the smelt, and 12% by the omnivore diet. Vitamin A was therefore consumed at 1770 µg/kg diet. Carotene was not detectable in the diet. Table 1 lists the estimated average quantities of diet components consumed daily by the bear.

Fischer NHsd/344 female rats (Harlan Sprague-Dawley, Indianapolis, IN) were maintained on the AIN semi-purified diet (89; ICN Nutritional Biochemicals, Cleveland, OH), with free access to tap water. Ten animals received a daily oral
Table 1. Estimated average quantities of diet and components thereof consumed daily by the bear.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
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<td>Total food</td>
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<tr>
<td>Metabolizable energy</td>
<td>8090 kcal</td>
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<tr>
<td>Crude protein</td>
<td>1788 g</td>
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<tr>
<td>Crude fiber</td>
<td>152 g</td>
</tr>
<tr>
<td>Fat</td>
<td>621 g</td>
</tr>
<tr>
<td>Vitamin A</td>
<td>77,669 IU</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>11,461 IU</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>72 mg</td>
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</table>

*Information provided by Dr. Bruce Watkins of Brookfield Zoo, Brookfield, IL.*

dose of 2 mg retinyl acetate in corn oil, while ten others received the vehicle only. On day 8 of dosing, tumor cells were transplanted into the napes of five animals from each group according to the following procedure. One gram of tumor was minced in 10 mL Liebovitz' L-15 medium (GIBCO, Grand Island, NY), containing gentamycin sulfate, streptomycin sulfate, and penicillin-G; 0.1 mL of the resulting suspension was injected into the loose skin of the nape. The dosed group was sacrificed on day 18 and the control group on day 19. Animals were anesthetized with diethyl ether and their livers and tumors were removed.
Transplantable rat mammary-tumor cells were acquired from the late Dr. John Balinsky, Dept. of Zoology, Iowa State University. This tumor line, induced by dimethylbenz(a)-anthracene, was kindly provided by Dr. Olga Greengard of Mt. Sinai Hospital, New York, NY. The tumor was maintained in Fischer NHsd/344 female rats, being transferred biweekly.

Preparation of Microsomes

With a Polytron apparatus, frozen polar-bear liver, which had been stored at \(-20^\circ C\) for about four months, was homogenized in 250 mM sucrose, buffered with 10 mM potassium phosphate, pH 7.4. The filtered homogenate was centrifuged at 13,000 \(\times g\) for 20 min in a Beckman JA-20 rotor at 4\(^\circ\)C. The supernatant solution was then centrifuged at \(8 \times 10^6 g\cdot\text{min}\) in a Beckman 80Ti rotor at 4\(^\circ\)C. Microsomes were washed once and suspended in 150 mM potassium phosphate buffer, pH 7.4, quick-frozen in liquid nitrogen, and stored at \(-70^\circ C\). Microsomal protein was measured by the Bradford dye-binding method (90).

Tumor samples were dissected free of necrotic tissue. With a motor-driven Teflon pestle, livers and tumors were homogenized in 250 mM sucrose, buffered with 10 mM potassium phosphate, pH 7.4. Microsomes were then prepared by the same
procedure as that used for bear liver. Microsomal protein was measured by absorbance at 280 nm, with BSA serving as standard.

**Measurement of Vitamin A**

Procedures for the extraction and spectrophotometric quantification of vitamin A were those previously described (91): about 1 g of frozen liver was mashed with sodium sulfate and its vitamin A was extracted with dichloromethane. The corrected absorbance of the extract at 325 nm was used in calculating the concentration of vitamin A in the liver. By the method of Furr et al. (92) retinyl esters and retinol were separated and quantified also by HPLC on a Waters Resolve 5-μm reversed-phase column, with a Waters 6000A pump used in conjunction with a Rheodyne injector, an LDC Spectromonitor III detector, which monitored absorbance at 325 nm, and a Shimadzu C-R2AX integrator. The eluting solvent, flowing at 1.5 mL/min, was acetonitrile/dichloromethane (80:20, v/v).

**Assay for Acyl CoA:Retinol O-Acyltransferase**

The assay for ARAT was based on Ross’ procedure (1). Microsomes were incubated with retinol, palmitoyl CoA, BSA, and dithiothreitol in 150 mM potassium phosphate buffer, pH
7.4, at a final volume of 0.5 mL. The reaction mixture without retinol was warmed in a shaking water bath at 37°C for 60 sec, at which time retinol was introduced in < 5 μL DMSO. Incubation lasted 15 min and 30 min for polar-bear and rat microsomes, respectively. Reaction was stopped by the addition of cold absolute ethanol at 2 mL per mL incubation solution, and the mixtures were then extracted with 2 x 1 mL hexane. The extracts were combined and the solvent was removed under argon. For subsequent analysis by HPLC the residue was dissolved in 75 μL n-decane/5-nonanone (2:1, v/v). Retinyl ester in the incubation extract was quantified by means of a standard curve relating peak area to the amount of ester injected. The amount of ester formed was the difference between that measured after incubation with active microsomes and that found after incubation with heat-inactivated microsomes. The HPLC system comprised a Waters 6000A pump in conjunction with a Waters WISP 710B autoinjector, a Shimadzu C-R3A Chromatopac integrator, and an ISCO V absorbance detector, monitoring at 325 nm. When analysis did not require the separation of retinyl esters, a Whatman Partisil ODS-2 reversed-phase column was used with methanol/tetrahydrofuran (90:10 or 80:20, v/v), flowing at 1.5 mL/min. Retinyl esters were separated on a Waters Resolve 5-μm reversed-phase column, with acetonitrile/dichloromethane (80:20, v/v) as the eluting solvent, also at a flow rate of 1.5 mL/min (92).
RESULTS AND DISCUSSION

As determined by HPLC, retinyl esters were present in this polar-bear liver at a concentration of 7880 μg retinol/g wet tissue, whereas free retinol was present at 170 μg/g. The total concentration of vitamin A, then, was 8050 μg/g, of which 98% was the ester form. By spectrophotometry the total vitamin-A concentration was somewhat lower (7400 μg/g). Both values, however, fall in the range of those previously published for the polar bear. Despite his having been raised in captivity, the bear in this investigation had hepatic vitamin-A reserves on a scale with those of his wild cousins. He clearly was fed generous amounts of vitamin A, though how his intake compares with that of wild bears cannot be assessed.

As shown in Table 2, retinyl palmitate was the predominant ester (37.3%), followed by retinyl oleate (20.9%), stearate (12.8%), and linoleate (7.7%). The pentadecanoate and heptadecanoate esters together accounted for 4.4% of the total. No other retinyl esters of odd-chain fatty acids were identified. Because all peak areas were proportionately smaller when absorbance was monitored at 280 or 450 nm, all peaks eluting after retinol were regarded as retinyl esters; no non-retinoid peaks were detected.

The predominance of retinyl palmitate in this sample of
Table 2. Retinyl ester composition of polar-bear liver

<table>
<thead>
<tr>
<th>Retinyl ester</th>
<th>μg retinol/g liver</th>
<th>Percent of total ester concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laurate</td>
<td>163</td>
<td>2.1</td>
</tr>
<tr>
<td>Myristate</td>
<td>155</td>
<td>2.0</td>
</tr>
<tr>
<td>Pentadecanoate</td>
<td>136</td>
<td>1.7</td>
</tr>
<tr>
<td>Palmitate</td>
<td>2936</td>
<td>37.3</td>
</tr>
<tr>
<td>Heptadecanoate</td>
<td>208</td>
<td>2.6</td>
</tr>
<tr>
<td>Stearate</td>
<td>1008</td>
<td>12.8</td>
</tr>
<tr>
<td>Oleate</td>
<td>1647</td>
<td>20.9</td>
</tr>
<tr>
<td>Linoleate</td>
<td>610</td>
<td>7.7</td>
</tr>
<tr>
<td>Unidentified (8 peaks)</td>
<td>1015&lt;sup&gt;C&lt;/sup&gt;</td>
<td>12.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> A weighed portion of frozen polar-bear liver was mashed with sodium sulfate and its retinol and retinyl esters were extracted into dichloromethane. The extract was analyzed by reversed-phase HPLC, the esters being separated on a Waters Resolve 5-μm C<sub>18</sub> column. Eluting solvent was acetonitrile/dichloromethane (80:20, v/v), flowing at 1.5 mL/min. The detector monitored absorbance at 325 nm.

<sup>b</sup> Ester concentration is expressed in terms of the retinol moiety.

<sup>c</sup> Largest peak represented 186 μg/g liver.

Polar-bear liver is consistent with the report of Futterman and Andrews (2), who found that ester to be predominant in the livers of all eight species they examined. In seven of the species, retinyl stearate and oleate followed palmitate in concentration, also seen in this bear liver. Retinyl linoleate, in contrast, was the fourth most concentrated ester in the bear liver while ranking fourth only in the frog liver in Futterman and Andrews' study; it was fifth to ninth in all
other species.

Dietary intake is an attractive explanation for the relatively larger concentration of retinyl linoleate in bear liver. Furr et al. (93) reported that the fatty-acid composition of retinyl esters in the liver reflects changes in dietary fatty acids. Among their observations was an increase in the proportion of retinyl linoleate in rats fed large amounts of trilinolein. Although Tomassi and Olson (94) saw no effect of changes in dietary fatty acids on retinyl esters, their experiments were shorter-term than those of Furr et al. (93). This question is discussed in Part III, pp. 91-92).

The rate of retinol esterification was not higher when microsomes were incubated with retinol and palmitic acid unless CoA and ATP were present also, evidence for classifying the enzyme activity as an acyltransferase. ARAT activity increased with retinol concentration (Fig. 1A), reaching a maximum at 80 μM of 821 pmol retinyl palmitate formed/min/mg microsomal protein. Because retinol was endogenous to the microsomes, retinyl ester was formed in the absence of exogenous retinol at a rate of 275 pmol/min/mg microsomal protein, or 33% of the maximum. The activity also rose with the concentration of palmitoyl CoA (Fig. 1B), reaching a plateau at 50–65 μM. As in the case with retinol, ester was formed in the absence of exogenous fatty-acyl CoA, at a rate of 250 pmol/min/mg microsomal protein.
Figure 1. Formation rate for retinyl palmitate as a function of retinol (A) and palmitoyl CoA (B) concentration. The reaction mixture comprised 15 μM BSA, 5 mM dithiothreitol, and polar-bear-liver microsomes at 300 μg protein/mL in 150 mM potassium phosphate buffer, pH 7.4. In A, palmitoyl CoA was present at 50 μM and in B, retinol was present at 80 μM. Incubation lasted 15 min. Each datum is the mean for duplicate trials, each vertical bar enclosing the range.
Whereas the largest increase in ARAT activity for polar-bear liver by BSA was only 46% (Fig. 2A), an increase of 89% was seen for rat-liver ARAT, but only when exogenous acyl CoA was present in the reaction mixture (Table 3). Increases much more dramatic than that for polar-bear ARAT also have been reported for ARAT from the small intestine of rats (55) and humans (56), although the dependence of ARAT from human liver (53) on BSA was not reported. Considering the analogous BSA-dependent transacylation of cholesterol, Lichtenstein and Brecher (79) proposed that BSA, by binding fatty-acyl CoA, may prevent the latter's detergent-like action on the microsomes or increase the substrate's accessibility to the enzyme. In accord with this hypothesis, the data in Table 3 show that BSA had no effect on reaction rate when the only acyl-CoA substrate available to ARAT was endogenous. As the concentration of palmitoyl CoA in incubations with polar-bear microsomes rose from 50 to 80 µM, the ratio of exogenous acyl CoA to BSA increased from 3.3:1 to 5.3:1, well above the ratio of 1:1 at which BSA binds oleoyl CoA (79). Polar-bear ARAT seems to be less sensitive to the detergency of palmitoyl CoA, the ratio of acyl CoA to BSA being about 3.3:1 before the reaction rate stopped responding to further increases in the concentration of palmitoyl CoA (Fig. 2A).

ARAT was not appreciably affected by dithiothreitol (Fig 2B), the reaction rate in its absence being 84% of the
Table 3. Stimulation of the activity of rat-liver ARAT by the addition of BSA or dithiothreitol (DTT) to the reaction mixture

<table>
<thead>
<tr>
<th>Additions to reaction mixture</th>
<th>Pmol retinol esterified/min/mg microsomal protein&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1:</td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>208</td>
</tr>
<tr>
<td>BSA (20 µM)</td>
<td>183</td>
</tr>
<tr>
<td>BSA (60 µM)</td>
<td>190</td>
</tr>
<tr>
<td>palmitoyl CoA (60 µM)</td>
<td>187</td>
</tr>
<tr>
<td>palmitoyl CoA (60 µM), BSA (20 µM)</td>
<td>303</td>
</tr>
<tr>
<td>palmitoyl CoA (60 µM), BSA (60 µM)</td>
<td>352</td>
</tr>
<tr>
<td>Experiment 2:</td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>palmitoyl CoA (60 µM)</td>
<td>ND</td>
</tr>
<tr>
<td>palmitoyl CoA (60 µM), DTT (2 mM)</td>
<td>181</td>
</tr>
<tr>
<td>palmitoyl CoA (60 µM), DTT (6 mM)</td>
<td>329</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values are means for duplicate trials, agreeing within 5%.

<sup>b</sup>Basic reaction mixture comprised rat-liver microsomes (300 µg protein/mL) and 52 µM retinol in 150 mM potassium phosphate buffer, pH 7.4, at a final volume of 0.25 mL. This mixture also included either 5 mM dithiothreitol (Experiment 1) or 20 µM BSA (Experiment 2).

<sup>c</sup>"ND" = "not detectable".

maximum. For rat-liver microsomes, however, dithiothreitol was a requirement for reaction (Table 3). Ross (1) reported that rat-liver ARAT was only 46% as active when dithiothreitol and BSA were absent from the reaction mixture as it was when they were present at 5 mM and 20 µM, respectively. Either the polar-bear enzyme is not dependent upon sulfhydryl groups or
Figure 2. Formation rate for retinyl palmitate as a function of BSA (A) and dithiothreitol (B) concentration. The reaction mixture comprised 80 μM retinol, 50 μM palmitoyl CoA, and polar-bear-liver microsomes at 300 μg protein/mL in potassium phosphate buffer, pH 7.4. In A, dithiothreitol was present at 5 mM and in B, BSA was present at 15 μM. Incubation lasted 15 min. Each datum is the mean for duplicate trials, each vertical bar enclosing the range
the groups themselves are not prone to oxidation under the conditions of this experiment.

Although Ross (1,66) reported some reaction rates for ARAT from rat liver on a par with the maximum reported here for the bear enzyme, a comparison of the plots of reaction rate as a function of retinol concentration reveals bear-liver ARAT to be the more active. The highest rate of esterification seen for rat-liver ARAT was about 400 pmol/min/mg microsomal protein, 49% of the maximal rate for its counterpart in the bear. ARAT activity for vitamin-A-normal rats is usually well below the rate of 821 cited for the bear (1,72,95; Table 1; Part II, p. 54). The highest reaction rate seen for ARAT from human liver was about 370 pmol/min/mg microsomal protein (53), or only 45% of the maximum for bear-liver ARAT.

In response to an excess of vitamin A, the liver expands its stores of the vitamin by accelerating retinol esterification. Such an increase in the tissue concentration of retinyl esters has been noted in lamb liver and kidney (57,96), chick liver (73), and rat liver (72). Inasmuch as ARAT activity is enhanced by larger-than-normal consumption of vitamin A (72,73), ARAT in polar-bear liver may be very active as the result of genetic programming or it may simply be in a state of perpetual enhancement because of the bear's constant large dietary supply of retinol. Whatever the reason for it, the unusually high activity of hepatic ARAT in the polar bear,
Table 4. ARAT activity in rat liver and mammary tumor before and after dosing with retinyl acetate

<table>
<thead>
<tr>
<th></th>
<th>Tumor-bearing</th>
<th>Tumor-free</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor</td>
<td>Liver</td>
</tr>
<tr>
<td>Control</td>
<td>12 ± 5</td>
<td>68 ± 17</td>
</tr>
<tr>
<td>Dosed</td>
<td>34 ± 23</td>
<td>280 ± 110</td>
</tr>
<tr>
<td>Dosed/Control</td>
<td>2.8</td>
<td>4.1</td>
</tr>
<tr>
<td>Value of $p$</td>
<td>&lt; 0.07</td>
<td>&lt; 0.025</td>
</tr>
</tbody>
</table>

All incubation mixtures comprised 20 μM BSA, 5 mM dithiothreitol, 100 μM retinol, 100 μM palmitoyl CoA, and microsomes at 400 μg protein/mL, in 150 mM potassium phosphate buffer, pH 7.4, at a final volume of 0.5 mL. Mean values ± standard deviations are reported for five animals, expressed as "pmol retinyl palmitate formed/min/mg microsomal protein". Each incubation, 30 min in length, was conducted in duplicate. Values of $p$ were calculated by means of Student’s t test.

A species whose reserves of vitamin A are naturally very large, bolsters the hypothesis that ARAT plays a physiological role in the storage of vitamin A.

Microsomes from rat mammary tumor also contain an enzyme activity that responds in vitro to the addition of retinol and palmitoyl CoA (Table 4). Thus, while vitamin A is undetectable in it (88; O. Amedee-Manesme and H. C. Furr, Dept. of Biochemistry and Biophysics, Iowa State University, Ames, Iowa, personal communication), mammary tumor tissue has the chemical machinery for synthesizing retinyl esters. However, the specific activity of ARAT in tumor microsomes was only 7% of that in microsomes from normal rat liver, and only
13% even after administration of vitamin A to the rats. ARAT activity was enhanced in this investigation not only in mammary tumor, but also in rat liver (Table 4), in accord with other reports (72,73).

As expected physiologically, then, ARAT activity in rat mammary tumor, a tissue that does not store vitamin A, is very low relative to that in liver, a tissue with substantial reserves of the vitamin. This observation and the fact that the activity of tumor ARAT was enhanced by administration of vitamin A to the animals (although the statistical significance is marginal) are additional evidence for ARAT's physiological role in vitamin-A storage. Moreover, the enhancement of ARAT activity by dosing with vitamin A may be a response to the toxicological threat posed by excess retinol in the liver.

Inasmuch as vitamin A is undetectable in rat mammary tumor, the presence of ARAT in this tissue has other implications. Given that ARAT activity, albeit very low, should be sufficient to form detectable amounts of retinyl ester in vivo, the tumor tissue may 1) catabolize vitamin A quickly, 2) be supplied with less vitamin A than normal tissues, or 3) not effectively absorb vitamin A from the blood. Any of these possibilities could lower the tumor's concentration of vitamin A to very low values.
ARAT activity in the liver of tumor-bearing rats was 60% lower than that in normal liver (Table 4). Administration of vitamin A not only enhanced the activity in liver but also equalized liver ARAT activities in tumor-bearing and tumor-free rats. Whereas the depression of ARAT activity in tumor-bearing rats must have resulted from some influence exerted by the tumor on the liver, the nature of the inhibition is not clear. In contrast, the activities of specific isozymes of hexokinase and pyruvate kinase in liver are higher in animals bearing this tumor (97). Whether the mechanism of in-vivo ARAT enhancement by vitamin A involves enzyme induction, stimulation, or removal of inhibition remains to be proven.

Although the data in this section do not preclude the possibility that more than one enzyme is involved, they strongly support the hypothesis that ARAT has a physiological role in the esterification of retinol in the liver. An acyl-CoA-independent retinol-acylating activity has been identified recently in microsomes from rat small intestine (15) and the properties of the enzyme differ from those published for small-intestinal ARAT (55, 56).

The acyl-CoA-independent activity, whose substrates are retinol bound to CRBP(II) and an unknown endogenous acyl donor, produces retinyl esters whose fatty-acid composition more nearly matches that of in-vivo retinyl esters than do esters formed by the ARAT-catalyzed reaction. Furthermore,
the $K_m$ of the acyl-CoA-independent activity is 0.2 μM (15), at least an order of magnitude lower than $K_m$ for ARAT from rat (55) or human (56) small intestine. Therefore, ARAT may operate in vivo whenever the concentration of retinol exceeds what the other acylating system can handle, although whether or not such a pair of enzymes exists in the liver is unknown.
PART II.

DISRUPTION OF MICROSOMES BY 13-\textit{cis}-RETINOIC ACID AND ITS INHIBITION OF RETINOL ESTERIFICATION AND BENZO(a)PYRENE HYDROXYLATION
INTRODUCTION

Conclusions from studies on the inhibition of ARAT *in vitro* by retinoids have been drawn without data on the influence of these compounds on other microsomal enzyme systems (60,62,77). Furthermore, not only has inhibition of hepatic ARAT by retinoids not been reported, but the most commonly used therapeutic retinoid, 13-cis-retinoic acid, has escaped attention altogether as a possible inhibitor of retinol esterification *in vitro*.

13-cis-Retinoic acid (13-c-RA), known also as "isotretinoin" (Accutane®), is a vitamin-A analog used as a drug in the treatment of recalcitrant acne (98). Among the side-effects of therapy with this drug are hyperlipidemia and a fall in the proportion of cholesterol in high-density lipoproteins (99-101). In rats, 13-c-RA not only raises the level of triacylglycerols in serum (102,103) but also changes the composition of both hepatic phospholipids (104,105) and microsomal fatty acids (105). Dosing rats with 7 µmol of retinyl acetate for 17 days also changed the fatty-acid composition both of total liver lipids and of retinyl esters (H. C. Furr and J. A. Olson, Dept. of Biochemistry and Biophysics, Iowa State University, Ames, Iowa, personal communication). Moreover, 13-c-RA both increases the concentration of lipid peroxides and decreases the level of α-
tocopherol in the plasma and liver of rats (106).

The site of the adverse effects of 13-c-RA may well be cellular membranes. For example, 13-c-RA disrupts mitochondrial membranes *in vitro* with a high degree of molecular specificity (107). Furthermore, all-trans-retinoic acid (all-t-RA) inhibits microsome-catalyzed lipid peroxidation (108), whereas 13-c-RA raises the activity of the microsomal Δ6-desaturase while lowering that of the Δ9-desaturase (105).

In testing for further interactions between 13-c-RA and membranes, three microsome-catalyzed reactions were selected: mannose-6-phosphate (M6P) hydrolysis, retinol esterification, and benzo(a)pyrene hydroxylation. Changes in the activity of mannose-6-phosphatase (M6Pase) are used as an index of the integrity of microsomal membranes (109-111). Because the active site of M6Pase is lumenal, an increase in the rate of M6P hydrolysis is interpreted as a greater membrane permeability to the substrate, reflecting membrane disruption. Vitamin-A metabolism is altered by 13-c-RA, in that the drug causes a fall in the concentration of serum retinol (102,103,106), and some patients on therapy with it have developed night blindness (112), a classic sign of vitamin A deficiency (113). Finally, the genetic modification of murine bone-marrow cells by benzo(a)pyrene, which is activated by hydroxylation, is prevented by vitamin A (114).
For the sake of comparison, \( N-(4\text{-hydroxyphenyl})- \) retinamide, retinyl \( \beta\text{-D-glucuronide} \), 15-methylretinol (each all-trans in configuration), and all-t-RA were also tested as in-vitro inhibitors of ARAT.
EXPERIMENTAL PROCEDURE

Materials

Retinoic acid, both all-trans and 13-cis, were purchased from Eastman Kodak Co. (Rochester, NY). Retinal, retinyl palmitate, palmitoyl CoA, DL-dithiothreitol, BSA (essentially globulin- and fatty-acid-free), benzo(a)pyrene, palmitic acid, taurocholic acid, and NADPH were supplied by Sigma Chemical Co. (St. Louis, MO). 3-Hydroxybenzo(a)pyrene was obtained from Midwest Research Institute (Kansas City, MO) and 3-[(3-cholamidopropyl)-dimethyl-ammonio]-l-propane sulfonic acid (CHAPS) from Calbiochem-Behring (La Jolla, CA). Barium mannose-6-phosphate (M6P) was purchased from Boehringer Mannheim (Indianapolis, IN). N-(4-hydroxyphenyl)retinamide were synthesized by Dr. Arun Barua of Iowa State University (115), as was retinyl B-glucuronide (116). 15-Methylretinol was prepared by the method of Tosukhowong and Olson (117), in which methylation was accomplished with methyl lithium. All other chemicals and solvents were of the highest quality commercially available. all-trans-Retinol was prepared and its concentration determined as before (Part I, p. 19). all-t- and 13-c-RA were dissolved in DMSO and the concentration of each was determined by absorbance at its \( \lambda_{\text{max}} \) (\( \lambda_{\text{max}} \) and \( \varepsilon \) for all-t- and 13-c-RA, respectively: 350 nm, 45,200 \( M^{-1} \text{cm}^{-1} \), and
354 nm, 39,800 M$^{-1}$ cm$^{-1}$). The stock solution of palmitoyl CoA was prepared and its concentration determined as before (Part I, p. 19). Benzo(a)pyrene was dissolved in acetone and 3-hydroxybenzo(a)pyrene in absolute ethanol; the concentration of the latter then was determined by absorbance at 380 nm ($\varepsilon = 30,800$ M$^{-1}$ cm$^{-1}$), after which the ethanol was removed under argon and the residue was redissolved in 1.0 N NaOH.

Retinoids and pyrenes were kept under argon, strictly out of white light, with gold fluorescent laboratory lights (Sylvania F40GO) serving as the only source of illumination.

Preparation of Microsomes

Fischer NHsd/344 female rats (Harlan Sprague-Dawley, Indianapolis, IN), 6–7 weeks old, were used upon arrival. Innovar-Vet$^R$ (Pitman-Moore, Washington Crossing, NJ) was injected i.p. at 0.06 mL/100 g body weight. Excised livers were rinsed, minced, and homogenized in 250 mM sucrose buffered with 15 mM potassium phosphate, pH 7.4. The homogenate first was centrifuged for 20 min at 13,000 x g in a Beckman JA-20 rotor at 4°C, and then the resulting supernatant solution at 7 x 10$^6$ g*min in a Beckman 80Ti rotor at 4°C; microsomes were washed once. The final pellet was suspended in 150 mM potassium phosphate, pH 7.4, or in 250 mM sucrose buffered with 10 mM Tris–maleate (pH 7.5 at 4°C).
suspension was distributed into capped microcentrifuge tubes, which were then submerged in liquid nitrogen and subsequently stored at -70°C. Microsomal protein was measured by the method of Smith et al. (118), wherein BSA served as standard.

Assay for Mannose-6-Phosphatase

Mannose-6-phosphatase activity was measured in microsomes by a method based on that of Arion et al. (111). At a final volume of 0.1 mL, microsomes were preincubated on ice for 15 min in 250 mM sucrose buffered with 5 mM imidazole, pH 7.5, with detergent at specified concentrations, and with or without BSA or rat serum. Substrate solution (0.4 mL) was then added and the mixture was incubated in a shaking water bath at 37°C for 30 min. The final reaction mixture comprised 10 mM M6P, 1 mM EDTA, 13 mM imidazole (pH 6.5), and 120 μg microsomal protein. Reaction was stopped by the addition of 2.0 mL 10% trichloroacetic acid, and, after brief centrifugation, inorganic phosphate was measured by the method of Fiske and SubbaRow (119).

Assay for Acyl CoA:Retinol O-Acyltransferase

The standard reaction mixture for ARAT assays comprised 20 μM BSA, 5 mM dithiothreitol, 70 μM palmitoyl CoA, rat-liver
microsomes, and all-trans-retinol in 150 mM potassium phosphate buffer, pH 7.4. Incubation, lasting 10 min, was performed in a shaking water bath at 37°C in the open air and under gold light. Retinoids were added in DMSO after the reaction mixture had been prewarmed for 2 min. Reaction was stopped by the addition of 1.0 mL cold absolute ethanol and the mixture was extracted with 1.5 mL hexane. The solvent was removed under argon and the residue was redissolved in 100 μL n-butyl acetate. Newly formed retinyl palmitate was separated from other retinyl esters and quantified by the reversed-phase HPLC system described before (Part I, p. 24).

Assay for Benzo(a)pyrene Hydroxylation

Hydroxylation of benzo(a)pyrene was assayed by a method modified after that of Nebert and Gelboin (120). The reaction mixture, with or without BSA at 1 mg/mL, contained 50 mM Tris-HCl buffer (pH 7.5 at 37°C), 3 mM MgCl₂, 720 μM NADPH, 64 μM benzo(a)pyrene, and microsomal protein at 271 μg/mL, in a final volume of 0.25 mL. With 13-C-RA the mixture was warmed in a shaking water bath at 37°C for 2 min, at which time benzo(a)pyrene was added in 2 μL acetone. Incubation lasted 30 min and was conducted in the open air under gold light. Reaction was stopped by the addition of 0.25 mL acetone, the mixture was extracted with 0.8 mL hexane, and the tube was
returned to the water bath for a 10-min incubation. A 0.25-mL aliquot of the organic phase was then extracted with 0.75 mL 1.0 N NaOH; the alkaline extract was removed and diluted with 2.0 mL 1.0 N NaOH. Fluorescence of the aqueous extract was measured with excitation at 396 nm and emission at 522 nm. The Spex fluorimeter was coupled to a Spex Datamate, with the photomultiplier tube water-cooled and the slit-width set at 1.25 mm. Measurements were corrected for the presence of 13-C-RA in the alkaline extract, and a standard curve was generated for 3-hydroxybenzo(a)pyrene fluorescence in 1.0 N NaOH.
RESULTS AND DISCUSSION

The activity of M6Pase in rat-liver microsomes was increased by 13-c-RA, 10% at 47 μM and 115% at 470 μM (Fig. 1). In contrast, at 470 μM, palmitate stimulated hydrolysis only 33%, CHAPS 16%, all-t-RA 9%, and taurocholate 0%.

Other amphiphiles also stimulate M6Pase in vitro (109-111,121). In those reports, however, the activity in untreated microsomes varies from 5% to 35% of the maximal activity reported in the presence of detergent, probably depending on the way in which microsomes were prepared and stored. Thus, in a comparison of these results, the concentration of detergent giving maximal stimulation of M6Pase is more meaningful than the degree of stimulation for a given microsome preparation. Table 1 summarizes the results of current and past studies. Clearly, 13-c-RA is among the most active amphiphiles tested. This observation is in accord

Table 1. Concentrations of amphiphiles that maximally stimulate mannose-6-phosphatase in rat liver

<table>
<thead>
<tr>
<th>Amphiphile</th>
<th>Concentration (mM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>13-cis-Retinoic acid</td>
<td>0.30-0.47</td>
<td>Present study</td>
</tr>
<tr>
<td>Oleoyl CoA</td>
<td>&gt; 0.30</td>
<td>110</td>
</tr>
<tr>
<td>Deoxycholate</td>
<td>3-3.8</td>
<td>110</td>
</tr>
<tr>
<td>Taurocholate</td>
<td>3.5-3.9</td>
<td>111</td>
</tr>
<tr>
<td>CHAPS0</td>
<td>3-4</td>
<td>109</td>
</tr>
</tbody>
</table>
Figure 1. Increase in mannose-6-phosphatase activity by amphiphilic compounds. Rat-liver microsomes (120 μg) were preincubated for 15 min at 0°C with 250 mM sucrose and 5 mM imidazole, pH 7.5, with 13-c-RA (□), all-t-RA (V), palmitate (○), taurocholate (△), or CHAPS (○), at a final volume of 0.1 mL. Substrate solution was then added and M6Pase was assayed. Microsomes used were free of inorganic phosphate. Incubations were conducted as singlets, and the data are typical for several trials.
Table 2. Protection by bovine serum albumin or rat serum against stimulation of mannose-6-phosphatase by 13-cis-retinoic acid

<table>
<thead>
<tr>
<th>Additions to incubation</th>
<th>Relative rate of M6P hydrolysis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Absolute increase</th>
<th>Relative increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NA</td>
</tr>
<tr>
<td>13-c-RA</td>
<td>2.14</td>
<td>114%</td>
<td>1.00</td>
</tr>
<tr>
<td>13-c-RA + BSA</td>
<td>1.83</td>
<td>83%</td>
<td>0.73</td>
</tr>
<tr>
<td>13-c-RA + rat serum</td>
<td>1.29</td>
<td>29%</td>
<td>0.25</td>
</tr>
</tbody>
</table>

<sup>a</sup>Rat-liver microsomes (120 µg protein) were preincubated for 15 min at 0°C with 13-c-RA at the specified concentrations, with or without BSA or rat serum at 2.3 mg protein/mL. At a final volume of 0.1 mL, preincubation solution also contained 250 mM sucrose buffered with 5 mM imidazole, pH 7.5. Substrate solution (0.4 mL) was then added and the final reaction mixture comprised 10 mM M6P, 1 mM EDTA, 13 mM imidazole (pH 6.5), and microsomal protein at 240 µg/mL. After a 30-min incubation at 37°C, 2.0 mL 10% trichloroacetic acid was added and inorganic phosphate was measured by the method of Fiske and SubbaRow (119). Values are the means of duplicate trials, agreeing within 5%.

<sup>b</sup>Absolute rate of 46 nmol inorganic phosphate released/min/mg microsomal protein.

<sup>c</sup>"NA" = "not applicable".

with that of Kon' et al. (107), that 13-c-RA (and all-trans-retinol), but not all-t-RA or particular synthetic retinoids, disrupted mitochondrial membranes at 525 µM.

BSA or rat serum, when present in the preincubation of microsomes with 13-c-RA at 470 µM, attenuated the increase in membrane permeability to M6P (Table 2). In the presence of BSA at 2.3 mg/mL, the increase in M6Pase activity was only 73% of the increase without BSA. Under the same conditions and at
the same total concentration of protein, rat serum was even more protective, the increase being only 25%. Inasmuch as BSA can bind retinoic acid (122), it may lower the amount of 13-c-RA free to interact with the membranes. In serum, however, RBP (123), as well as albumin, lipoproteins, and possibly other serum components, may offer additional protection.

Retinol esterification was inhibited in vitro by 13-c-RA (Fig. 2). Whereas 13-c-RA and all-t-RA behaved differently with respect to M6Pase (Fig. 1), they both inhibited ARAT, and to about the same extent (Fig. 2), even over a range of concentrations (Fig. 3). Thus, ARAT inhibition was independent of geometric configuration at C-13, while membrane disruption was much greater with the cis form. Data were fit to the Michaelis-Menten equation by means of the computer program of Oestreicher and Pinto (124). The calculated $V_{max}$ (319 pmol/min/mg microsomal protein without inhibitor) fell 26% and 21% in the presence of all-t-RA and 13-c-RA, respectively. Without inhibitor, the value of $K_m$ was 19.9 μM, whereas it was 16.5 μM with all-t-RA and 16.4 μM with 13-c-RA (Fig. 2A). A Hanes plot of the experimental data (Fig. 2B) gave similar results. From these data, then, the mechanism of inhibition is obscure, ostensibly a mixture of competitive and non-competitive interactions.

At 32 μM retinol, the inhibition of ARAT rose progressively from 13% at 89 μM 13-c-RA to 83% at 440 μM (Fig.
Figure 2. Inhibition of retinol esterification by retinoic acid. Rat-liver microsomes (175 μg protein) were incubated with retinol in the absence (■) or presence of all-t-RA (●) or 13-c-RA (△) at 50 μM. (A) Relative rate of retinol esterification vs. retinol concentration. Reaction mixtures, at a final volume of 0.5 mL, contained 20 μM BSA, 5 mM dithiothreitol, 70 μM palmitoyl CoA, and retinol in 150 mM potassium phosphate buffer, pH 7.4. Incubation lasted 10 min. (B) Hanes plot ([S]/v vs. [S]) of the data in A. Incubations were conducted as singlets, but the correlation coefficient for each line in B is > 0.99
Figure 3. Inhibition of retinol esterification by retinoic acid at a fixed concentration of retinol. Rat-liver microsomes were incubated with either all-\( t-\)RA (□) or 13-\( c-\)RA (●), with retinol at 32 \(\mu\)M. Incubation conditions were otherwise the same as described for Fig. 2.
This inhibition follows a similar course as the stimulation of M6Pase (Fig. 1), with 50% ARAT inhibition and 50% M6Pase stimulation occurring at about the same concentration of 13-c-RA (200 μM). Furthermore, ARAT inhibition and M6Pase stimulation reached plateaus in the same range of 13-c-RA concentrations. The degree of inhibition increased slightly with the time of preincubation of microsomes with 13-c-RA. With 13-c-RA at 222 μM, ARAT was inhibited 51% without preincubation, whereas with 12-min preincubation the inhibition was 61%. These observations, along with the lack of a kinetically distinct mechanism of inhibition, are consistent with the notion that loss of membrane integrity played a large part in the inhibition of ARAT by 13-c-RA in vitro.

Whatever the mechanism of ARAT inhibition, 13-c-RA in vivo is not likely to reach a concentration necessary for substantial inhibition in these in-vitro experiments, since the drug is not stored in the liver (125) and since the in-vitro concentration of 13-c-RA has been shown merely to double upon massive dosing (106). The underlying biochemical problems with night vision reported in patients on therapy with 13-c-RA (112) probably do not involve ARAT in the liver. This conclusion is reinforced by studies demonstrating that the level of neither retinyl palmitate (106) nor total vitamin A (103) in rat liver is affected by dosing with 13-c-RA.
Figure 4. Inhibition of retinol esterification and benzo(a)pyrene hydroxylation by 13-cis-retinoic acid. All reaction mixtures, at a final volume of 0.25 mL, contained rat-liver microsomes at 271 μg protein/mL. (A) Relative rate of retinol esterification vs. 13-C-RA concentration. Retinol was present at 32 μM. ARAT was assayed otherwise as described for Fig. 2. (B) Relative rate of benzo(a)pyrene hydroxylation (BP) vs. 13-C-RA concentration. BSA was either absent (●) or present (○) at 1 mg/mL. Each datum is the mean for duplicate trials, each vertical bar enclosing the range.
Benzo(a)pyrene hydroxylation, presumably catalyzed by aryl hydrocarbon hydroxylase (AHH) in these rat-liver microsomes, also was slower in the presence of 13-c-RA (Fig. 4B). With a rate of 120 pmol/min/mg microsomal protein without inhibitor, the inhibition was 31% and 62% with 13-c-RA at 44 and 440 µM, respectively. In contrast, Hill and Shih (126) reported that AHH in mouse- or hamster-liver microsomes was not inhibited in vitro by 13-c-RA. In that study, however, 13-c-RA was present in the reaction mixture at 50 µM, at which concentration none of the retinoids tested inhibited AHH by more than 15%. Fig. 4B shows that inhibition was 35-40% at 50 µM 13-c-RA in the present study. Moreover, Hill and Shih included BSA in the reaction mixture at 1 mg/mL; as seen in Fig. 4B, though, BSA did not attenuate the inhibition in this system.

Benzo(a)pyrene hydroxylation was inhibited by lower concentrations of 13-c-RA (< 100 µM) than was retinol esterification. Therefore, the apparent inhibition of AHH in vitro may bear greater physiological significance, although in one study the administration of 13-c-RA to rats did not change hepatic AHH activity subsequently assayed in vitro (127). Retinoids not only protect against damage by benzo(a)pyrene (114), but they also inhibit mixed-function oxidases (126), TCDD-induced lipid peroxidation (128), mutagenesis by quinoline (129), and formation of aflatoxin B₁-DNA adducts (130).
In smaller-scale experiments, two other retinoids also inhibited ARAT in vitro. Data for inhibition by \( N\)-(4-hydroxyphenyl)retinamide (HPR) and 15-methylretinol (MeROL) are shown in Fig. 5. For each retinoid, the linear transformation of the Michaelis-Menten equation giving the highest correlation coefficients was used. Inhibition by HPR displayed a competitive component (Fig. 5A), whereas inhibition by MeROL was strongly uncompetitive (Fig. 5B). In contrast, retinyl \( \beta\)-D-glucuronide had no effect on ARAT in vitro at < 50 \( \mu \)M. Thus, ARAT's active site seems accessible to HPR but inaccessible to MeROL. Consistent with this conclusion, the data in Table 3 show that MeROL is not

**Table 3. Ineffectiveness of 15-methylretinol as a substrate for ARAT**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (( \mu )M)</th>
<th>Relative rate^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinol</td>
<td>19</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>1.00</td>
</tr>
<tr>
<td>15-Methylretinol</td>
<td>15</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>0.08</td>
</tr>
</tbody>
</table>

^aIn 150 mM potassium phosphate buffer, pH 7.4, rat-liver microsomes (175 \( \mu \)g protein) were incubated with 20 \( \mu \)M BSA, 5 mM dithiothreitol, and 75 \( \mu \)M palmitoyl CoA, with either retinol or 15-methylretinol, at a final volume of 0.5 mL. Incubation, at 37°C, lasted 10 min; reaction was stopped with ethanol, the mixture was extracted with hexane, and the extract was analyzed by reversed-phase HPLC. Values are means for duplicate trials, agreeing within 5%.
Figure 5. Inhibition of ARAT in rat-liver microsomes by $N$-(4-hydroxyphenyl)retinamide and 15-methylretinol. (A) Lineweaver-Burk plot of data for inhibition by HPR. HPR was either absent ($\Delta$) or present at 50 (●) or 100 (○) μM. (B) Hanes plot of data for inhibition by MeROL. MeROL was either absent ($\Delta$) or present at 23 (●) or 46 (○) μM. Incubation conditions were otherwise the same as described for Fig. 2.
appreciably esterified by ARAT \textit{in vitro}. These data lend further support to the physiological importance of ARAT \textit{in vivo} in light of the report by Tosukhowong and Olson (117), showing the absence of significant esterification and storage of MeROL in the liver.

Inasmuch as 13-c-RA disrupts membranes in the same range of concentrations at which it inhibits ARAT and AHH, any retinoid under evaluation as an \textit{in-vitro} inhibitor of microsomal enzymes should be evaluated also as a potential disruptor of membranes, especially at high concentrations. Thus, conclusions regarding the mechanism, and hence physiological relevance, of the \textit{in-vitro} inhibition of microsomal enzymes by retinoids must consider the possibility that non-specific membrane disruption occurs simultaneously.

Clearly, then, at least one therapeutic retinoid capable of inhibiting ARAT \textit{in vitro} does so non-specifically, also inhibiting AHH and disrupting the microsomal membranes. In a physiological context, the ability of 13-c-RA to disrupt microsomes \textit{in vitro} at concentrations where other amphiphiles have little or no effect should serve as a starting point for investigations into the mechanism of this drug's side-effects.
PART III.

SPECIFICITY OF ACYL COENZYME A:RETINOL O-ACYLTRANSFERASE TOWARD ITS FATTY-ACYL-CoA SUBSTRATE
INTRODUCTION

As early as 1940 it was shown by Gray et al. (131) that, irrespective of its form, vitamin A fed to a rat was stored in the liver esterified with a small variety of fatty acids. In longer-term feeding experiments, however, Gray and Cawley (132) identified the predominant ester of vitamin A in rat liver as that of palmitic acid, with the range of fatty acids smaller than in the previous study. Subsequent reports confirmed these findings. As analyzed by Futterman and Andrews (2), the retinyl-ester fraction of the livers from eight species, human, rat, calf, sheep, rabbit, cat, frog, and trout, was mostly retinyl palmitate. The trio of palmitate, stearate, and oleate esters, moreover, accounted for no less than 70% of the total retinyl-ester concentration in all species except the frog, wherein they represented 64%. The predominance of retinyl palmitate in rat liver has been confirmed by Goodman et al. (22), Bhat and LaCroix (78), and Furr et al. (92). Although no retinyl oleate was detected in rat liver by Bhat and LaCroix (78), the palmitate/stearate/oleate trio accounted for 71% of the retinyl esters in polar-bear liver, with retinyl palmitate once again predominant (Part I, p. 26, Table 2). Retinol esterification, then, may be a fatty-acid-specific process.
Surprisingly, Tomassi and Olson (94) saw no difference in the fatty-acid composition of hepatic retinyl esters among rats fed either 10% corn, linseed, or coconut oil for ten days. In a longer-term set of experiments, however, Furr et al. (93) fed rats synthetic simple triacylglycerols at 8% in the diet. Differences in the fatty-acid composition of dietary fat were reflected in the fatty-acid composition not only of retinyl esters in the liver but of total liver lipids as well. Thus, if retinol esterification is fatty-acid-specific, it can nonetheless accommodate gross changes in the fatty acids supplied as substrates.

ARAT from various tissues uses several fatty-acyl-CoA thioesters as substrates, those tested being the stearoyl (1,56,58,59), oleoyl (1,55,56,58,59), linoleoyl (60), lauroyl (1,58), octanoyl (1,58), and, of course, palmitoyl (1,53,55,56,58-60,72,77) derivatives. No clear specificity for palmitoyl CoA has been demonstrated in ARAT from any tissue, although for liver (1) and small intestine (55,56) this derivative gives somewhat higher reaction rates. In fact, oleoyl and stearoyl CoA were better substrates for ARAT from lactating rat mamma (58), and were just as effective as palmitoyl CoA in the case of rat testis (59).

Ideally, defining the specificity of an enzyme in vitro would be a simple matter of 1) incubating the enzyme with various competing substrates, present together in the reaction
mixture at equimolar concentrations, and then 2) calculating the ratio of $k_{\text{cat}}$ to $K_m$ for each, inasmuch as this ratio is regarded as the true measure of a substrate's comparative effectiveness (133). However, fatty-acyl-CoA thioester derivatives are endogenous to rat-liver microsomes (134), and, as expected, they can serve as substrates for ARAT in in-vitro incubations (1).

Because a net-synthesis assay for ARAT is used in the present investigation, approaches to the question of ARAT's in-vitro specificity toward its acyl-CoA substrate must address the complication of endogenous acyl CoA. Moreover, in previous experiments microsomes have been incubated with the various thioesters present singly, rather than together in substrate-competition studies. Questions about the complication of acyl CoA hydrolases, which may compete for substrate, and BSA, which binds oleoyl CoA in vitro (79), must also be settled. Finally, data on CoA thioesters of most polyunsaturated and odd-chain fatty acids as substrates for ARAT are absent from the literature, even though fatty-acyl groups of these kinds have been found in hepatic retinyl esters in vivo (2,22,78,92; Part I, p. 26, Table 2).

The research described in this section considers these issues in defining the specificity of ARAT in vitro. For comparison, the specificity of ARAT from polar-bear liver also is examined.
EXPERIMENTAL PROCEDURE

Materials

Retinal, retinyl palmitate, fatty-acyl-CoA thioester derivatives, DL-dithiothreitol, and BSA (essentially globulin- and fatty-acid-free) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were of the highest quality commercially available. all-trans-Retinol was prepared and its concentration determined as before (Part I, p. 19). Each fatty-acyl-CoA derivative was dissolved in 150 mM potassium phosphate buffer, pH 7.4, and its concentration was determined by absorbance at 259.5 nm, with ε = 15,400 M⁻¹ cm⁻¹. Solutions of each derivative were quick-frozen in liquid nitrogen and stored at −20°C, the unsaturated thioesters being sealed under argon.

Preparation of Microsomes

As before, differential centrifugation was used for preparing liver microsomes from rat (Part II, p. 44) and polar bear (Part I, p. 22). After being washed once, microsomes were suspended in 150 mM potassium phosphate, pH 7.4. The final microsome suspension was distributed into capped microcentrifuge tubes, which were then submerged in liquid
nitrogen and subsequently stored at $-70^\circ\text{C}$. Microsomal protein was measured by the Bradford dye-binding method (90), with BSA serving as standard.

**Assay for Acyl CoA:Retinol O-Acyltransferase**

The standard reaction mixture for ARAT assays comprised 20 $\mu$M BSA, 5 mM dithiothreitol, rat-liver microsomes, all-trans-retinol, and fatty-acyl-CoA thioesters in 150 mM potassium phosphate buffer, pH 7.4. Incubation, extraction, and HPLC analysis were performed as before (Part II, p. 46).

**General Protocol**

Attempts to define the specificity of ARAT *in vitro* employed two experimental approaches. By virtue of their design and rationale, which are described here, the results of these two approaches should be mutually consistent.

In the first approach, microsomes were incubated with the exogenous fatty-acyl-CoA thioester derivatives to be tested as substrates, all together and equimolar in the reaction mixture. An apparent rate of formation was then calculated for each retinyl ester. However, for some retinyl esters this apparent rate included contributions from unknown amounts of acyl CoA endogenous to the microsomes. Therefore, a control
incubation, revealing how much of the apparent rate was attributable to endogenous substrate, was conducted for each retinyl ester.

The control incubation contained all exogenous thioesters except the one whose endogenous contribution was being determined. For a given retinyl ester, this control rate was interpreted as the endogenous substrate's contribution to the rate observed in incubations containing all the exogenous acyl-CoA derivatives. Correcting for endogenous acyl CoA, then, consisted of subtracting the control rate from the apparent rate:

\[ v_{\text{corrected}} = v_{\text{apparent}} - v_{\text{control}} \]

where "\( v_{\text{apparent}} \)" is the rate observed with all exogenous acyl-CoA derivatives present in the reaction mixture, and "\( v_{\text{control}} \)" is the rate with all but the corresponding acyl-CoA derivative present.

Of course, omitting an acyl-CoA derivative from the reaction mixture would have lowered the total concentration of exogenous fatty-acyl CoA. Therefore, the total concentration of fatty-acyl CoA in the control incubations and those containing all exogenous derivatives were equalized. For example, rat-liver microsomes were incubated with 11
thioesters, each present at 5 μM, for a total exogenous concentration of 55 μM.

The control incubation for, say, retinyl palmitate contained all exogenous derivatives except palmitoyl CoA, for a total exogenous concentration of only 50 μM. Any endogenous palmitoyl CoA in the control, then, would be competing with less total exogenous substrate than it would be in the incubation containing all thioesters, making the control rate deceptively high. Therefore, the concentration of each thioester in the control incubation for retinyl palmitate, from which palmitoyl CoA had been omitted, was made 5.5 μM to restore the total concentration of exogenous acyl CoA to 55 μM. Only when this adjustment is made are the control incubations valid.

In the second approach to the problem of specificity, microsomes were incubated with a variety of exogenous fatty-acyl-CoA derivatives, which had been added together and in equimolar amounts, at increasing total concentration. This approach assumes that as the total concentration of exogenous acyl CoA increases, the contribution from endogenous substrate should become less quantitatively important. Thus, a comparison of relative reaction rates at saturating concentrations of exogenous acyl CoA should provide insight into the specificity of ARAT in vitro without correcting for endogenous substrate.
Because of the limiting amount of sample available, experiments on the specificity of polar-bear-liver ARAT did not include control incubations for each retinyl ester, as in the first approach used for rat-liver ARAT. Instead, microsomes were incubated under three conditions: 1) in the absence of all exogenous acyl CoA, 2) in the presence of exogenous saturated derivatives, and 3) in the presence of a mixture of saturated and unsaturated derivatives. The "corrected" formation rate for a given retinyl ester was calculated by subtracting the rate when all exogenous substrate was absent from the rate when they were present.
RESULTS AND DISCUSSION

Fatty-acyl-CoA thioesters are endogenous to microsomes (1,134), whether they associate with the membranes artifactually during cell fractionation or exist as such in vivo. Because calculating $K_m$ for an acyl-CoA substrate requires knowing its concentration in the reaction mixture, either the endogenous substrates had to be removed or their relative concentrations determined.

In an effort to destroy the endogenous acyl CoA, microsomes were preincubated at 37°C with hydroxylamine, which reacts with acyl-CoA thioesters to give hydroxamates (65). Ross (1) was the first to use this approach for the purpose of removing endogenous acyl CoA from rat-liver microsomes. Inasmuch as adding palmitoyl CoA to reaction mixtures containing hydroxylamine-treated microsomes stimulated ARAT activity, ARAT in microsomes so treated was still active even though much of the endogenous acyl CoA had presumably been destroyed.

In the present study, however, conditions for treatment with hydroxylamine that were required for rendering the endogenous acyl CoA negligible also resulted in a substantial loss of ARAT activity. So great was the loss, in fact, that when acyl-CoA thioesters were present together in incubations,
the reaction rate for each was too low to be measured accurately.

Direct measurement of the endogenous acyl CoA also did not seem feasible. Whereas many methods for the extraction and analysis of acyl-CoA thioesters exist for various tissues and subcellular organelles (135-140), no technique for the extraction of these compounds from microsomes has been published. Because efforts to develop such a technique might have proven expensive and time-consuming, the approaches to defining the specificity of ARAT described under "General Protocol" were designed to circumvent the problem of endogenous substrate without compromising the quality of the data.

When rat-liver microsomes were incubated with retinol in the absence of exogenous acyl CoA, six retinyl esters were formed: retinyl laurate, myristate, palmitate, stearate, oleate, and linoleate (Table 1). These rat-liver microsomes, then, ostensibly contained the lauroyl, myristoyl, palmitoyl, stearoyl, oleoyl, and linoleoyl derivatives of CoA.

Although retinyl laurate and arachidonate do not co-elute with the HPLC system used here, the difference between their retention times is so small that these esters are identifiable only when both are present in a given run, in which case two clearly distinguishable peaks are observed. Inasmuch as arachidonic acid is much more abundant in rat liver than is
Table 1. Comparison of formation rates for retinyl esters synthesized in vitro by rat-liver ARAT with their concentrations in vivo

<table>
<thead>
<tr>
<th>Retinyl ester</th>
<th>Percentage of total rate$^{a}$</th>
<th>Reported percentage of total hepatic retinyl-ester concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>with all exogenous acyl CoA$^{b}$</td>
<td>with no exogenous acyl CoA$^{c}$</td>
</tr>
<tr>
<td>Laurate</td>
<td>6.0</td>
<td>3.8</td>
</tr>
<tr>
<td>Tridecanoate</td>
<td>11.0</td>
<td>ND$^{i}$</td>
</tr>
<tr>
<td>Myristate</td>
<td>10.3</td>
<td>4.9</td>
</tr>
<tr>
<td>Pentadecanoate</td>
<td>11.0</td>
<td>ND</td>
</tr>
<tr>
<td>Palmitate</td>
<td>12.0</td>
<td>38.2</td>
</tr>
<tr>
<td>Heptadecanoate</td>
<td>13.3</td>
<td>ND</td>
</tr>
<tr>
<td>Stearate</td>
<td>10.4</td>
<td>38.5</td>
</tr>
<tr>
<td>Arachidate</td>
<td>9.7$^{k}$</td>
<td>ND</td>
</tr>
<tr>
<td>Palmitoleate</td>
<td>NT</td>
<td>ND</td>
</tr>
<tr>
<td>Oleate</td>
<td>14.2</td>
<td>8.8</td>
</tr>
<tr>
<td>Linoleate</td>
<td>1.7</td>
<td>5.8</td>
</tr>
<tr>
<td>Linolenate</td>
<td>NT</td>
<td>ND</td>
</tr>
<tr>
<td>Arachidonate</td>
<td>0.6</td>
<td>ND</td>
</tr>
</tbody>
</table>
 Reaction mixtures comprised rat-liver microsomes at 237 μg protein/mL, 20 μM BSA, 5 mM dithiothreitol, 37 μM retinol, and fatty-acyl-CoA thioester derivatives in 150 mM potassium phosphate buffer, pH 7.4. Incubation, at 37°C, lasted 10 min; reaction was stopped with ethanol, the mixture was extracted with hexane, and the extract was analyzed by reversed-phase HPLC. Values are means for duplicate trials, agreeing within 5%.

Eleven exogenous fatty-acyl-CoA thioester derivatives (lauroyl, tridecanoyl, myristoyl, pentadecanoyl, palmitoyl, heptadecanoyl, stearoyl, arachidoyl, oleoyl, linoleoyl, and arachidonoyl) were included in the reaction mixture, each at 5 μM. See "General Protocol" for details; this rate is the same as "corrected".

No exogenous fatty-acyl CoA was present in the reaction mixture.

Mean for rats identified in the report as "D" through "K".

Ref. 92 was consulted in conjunction with a personal communication from the first author, H. C. Furr, Dept. of Biochemistry and Biophysics, Iowa State University, Ames, Iowa.

"NR" = "not reported".

The authors note that "small quantities of laurate, myristoleate, and several unidentified components were present".

Retinyl laurate and arachidonate co-eluted in this system.

"ND" = "not detected".

Retinyl myristate and palmitoleate co-eluted in this system.

"NT" indicates that the corresponding acyl CoA derivative was "not tested" as a substrate.
lauric acid (141,142), what has been called retinyl laurate in these studies, may, in reality, be the arachidonate ester.

Finding thioesters of CoA with $C_{16}$ and $C_{18}$ fatty acids endogenous to the microsomes is expected. These compounds are substrates for the enzymes in the endoplasmic reticulum that elongate palmitic acid to stearic and desaturate stearic acid to oleic, as well as those that synthesize arachidonic acid from linoleic (143). However, the presence of CoA thioesters of lauric and myristic acids is puzzling inasmuch as these two fatty acids occur in hepatic lipids in very small amounts relative to other fatty acids (141,142). Even in rats maintained on diets 10% in coconut oil, which is rich in lauric and myristic acids, the concentration of these acids in lipids of the liver was remarkably low (141).

Indeed, finding arachidonoyl CoA in the microsomes along with linoleoyl CoA would be consistent with the role of endoplasmic reticulum in the elongation and desaturation of fatty acids. Nonetheless, the retinyl-laurate peak has been so identified primarily because, as also shown in Table 1, it was formed by ARAT at a 10-fold higher rate than was retinyl arachidonate when rat-liver microsomes were incubated with the corresponding acyl-CoA thioesters. However, whether the peak is retinyl laurate or arachidonate, it represents less than 1% of the total retinyl-ester concentration in vivo (Table 1).
Retinyl myristate and palmitoleate have similar retention times also. Although what has been identified as retinyl myristate may have been retinyl palmitoleate, both of these esters have been found in the liver, and in larger amounts than retinyl laurate or arachidonate (Table 1).

Table 1 shows the results of experiments in which exogenous fatty-acyl-CoA thioesters were present together at equimolar amounts in the reaction mixture with rat-liver microsomes. Corrected for contributions from endogenous substrate, the percentages of the total rate of reaction for the various derivatives are listed as the first column of numbers. Clearly, all tested thioesters of saturated fatty acids from C₁₂ to C₂₀ were effective substrates for ARAT in vitro, although the rate for lauroyl CoA was only about 45% of the maximum in the saturated series, observed for heptadecanoyl CoA. Whereas oleoyl CoA was the most effective, the thioesters of linoleic and arachidonic acids were poor substrates for ARAT.

Whereas lauroyl CoA was only 50% as effective a substrate as palmitoyl CoA, Ross (1) reported it to be 92% as effective. Furthermore, the reaction rates for stearoyl and oleoyl CoA were 87% and 118%, respectively, of that for palmitoyl CoA in the present study, in contrast to 71% and 89%, respectively, in Ross' (1) experiments. Octanoyl CoA, not tested in the present study, gave a rate only 58% of that for palmitoyl CoA.
In explaining these discrepancies, two differences in design between the two experiments must be noted: 1) Ross (1) incubated microsomes with only one CoA derivative at a time, and 2) with each derivative at a concentration roughly twice the total concentration used in the present study; these differences in design may have translated into significant differences in results.

Reported critical micelle concentrations for these derivatives vary enormously with chain length from 7.5 μM for stearoyl CoA to 1130 μM for lauroyl CoA (144–145), and with ionic strength and the nature of the buffer (145). Whereas micelles of these CoA thioesters were presumably homogeneous in Ross' (1) reaction mixtures, in the present study they were likely to be mixed, though uniform in both size and composition. Accessibility of the fatty–acyl–CoA substrate to ARAT, therefore, may have been different in Ross' (1) study than in the present one.

An additional complication lies in the ability of BSA to bind oleoyl CoA in vitro (79), and perhaps other such thioesters also. While micelles undoubtedly differed between these experiments, binding of acyl CoA to BSA also may have differed, affecting substrate accessibility further. The kinetics of enzyme–catalyzed reactions for which acyl CoA is a substrate and BSA is included in the reaction mixture are inherently uncertain because studies on the partitioning of
Figure 1. Relative formation rates for retinyl esters at increasing concentration of total acyl CoA. Rat-liver microsomes were incubated at 37°C with lauroyl (12:0), myristoyl (14:0), palmitoyl (16:0), stearoyl (18:0), and oleoyl (18:1) CoA, added together and in equimolar amounts. Reaction mixture contained 20 μM BSA, 5 mM dithiothreitol, 48 μM retinol, and microsomal protein at 350 μg/mL. Incubation lasted 10 min. Each datum is the mean of duplicate trials, each vertical bar enclosing the range.
Total Concentration of Exogenous Acyl CoA (μM)

Percentage of Total Esterification Rate

- 12:0
- 14:0
- 16:0
- 18:0
- 18:1

0 20 50 80 110 140

0 10 20 30 40 50
acyl-CoA thioesters among the monomer, micelle, and BSA-bound forms are absent from the literature (146).

In the second approach to defining the specificity of ARAT, microsomes were incubated with five exogenous acyl-CoA derivatives, present together and in equimolar amounts, at increasing total concentration. As seen in Fig. 1, the palmitate and stearate esters of retinol were formed in greatest quantity when exogenous acyl CoA was absent from the reaction mixture, in general agreement with Table 1. As the total concentration of exogenous acyl CoA increased to apparent saturation (Fig. 2), endogenous substrate was progressively diluted. The rates for palmitoyl, stearoyl, and oleoyl CoA tended to equalize, while those for lauroyl and myristoyl CoA stayed lower (Fig. 1).

This approach was used also for testing polyunsaturated derivatives as substrates for ARAT. Microsomes were incubated with palmitoyl, stearoyl, oleoyl, linoleoyl, linolenoyl, and arachidonoyl CoA under the same conditions as described for Fig. 1. Whereas the formation rates for retinyl palmitate, stearate, and oleate tended to equalize as the total concentration of exogenous acyl CoA increased, the sum of the formation rates for retinyl linoleate, linolenate, and arachidonate was less than 1% of the total rate at any concentration.
Figure 2. Rate of retinyl ester formation as a function of the concentration of exogenous acyl CoA. Details are the same as those for Fig. 1.
Picomoles Total Retinyl Ester Formed
min⁻¹·mg Microsomal Protein⁻¹

Total Concentration of Exogenous Acyl CoA (μM)
Despite differences in experimental design, the fundamental conclusions emerging from the two approaches to the question of specificity in the present study are the same: 1) even under conditions of substrate competition, ARAT from rat liver can transacylate retinol with a variety of fatty-acyl-CoA derivatives, saturated, monounsaturated, even- or odd-chain, and 2) CoA thioesters of polyunsaturated fatty acids are poor substrates.

Conclusions from these specificity studies assume that the activity of other microsomal enzymes for which fatty-acyl-CoA thioesters are substrates is negligible under the conditions of these experiments. Inasmuch as retinol was present in the system in great excess over all other reactants except water, the only potentially competing reaction was hydrolysis of the acyl-CoA thioesters. Fortunately, directly measuring the hydrolysis to assess its quantitative importance was unnecessary inasmuch as Mg$^{2+}$-ion could be used to inhibit hydrolase activity in the reaction mixtures (147,148).

Neither the total rate of retinol esterification nor the percentage thereof for a given acyl-CoA thioester was affected by Mg$^{2+}$ (Table 2). Microsomal fatty-acyl-CoA hydrolases, then, did not confound the results of specificity experiments by competing with ARAT for substrate in vitro. Although only saturated derivatives were used to generate the data in Table 2, the dismissal of microsomal thioesterases as competing
Table 2. Effect of inhibiting microsomal acyl CoA hydrolase activity on relative formation rates of retinyl esters

<table>
<thead>
<tr>
<th>Retinyl ester</th>
<th>(-\text{Mg}^{2+})</th>
<th>(+\text{Mg}^{2+})</th>
<th>(-\text{Mg}^{2+})</th>
<th>(+\text{Mg}^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laurate</td>
<td>ND</td>
<td>ND</td>
<td>6.4</td>
<td>6.0</td>
</tr>
<tr>
<td>Tridecanoate</td>
<td>ND</td>
<td>ND</td>
<td>9.2</td>
<td>9.5</td>
</tr>
<tr>
<td>Myristate</td>
<td>ND</td>
<td>ND</td>
<td>15.0</td>
<td>14.6</td>
</tr>
<tr>
<td>Palmitate</td>
<td>47.5</td>
<td>47.7</td>
<td>38.8</td>
<td>38.5</td>
</tr>
<tr>
<td>Stearate</td>
<td>49.3</td>
<td>48.8</td>
<td>30.6</td>
<td>31.4</td>
</tr>
<tr>
<td>Oleate</td>
<td>3.2</td>
<td>3.5</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Linoleate</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

In potassium phosphate buffer, pH 7.4, at a final volume of 0.5 mL, rat—liver microsomes (150 μg protein) were incubated with 20 μM BSA, 5 mM dithiothreitol, and 52 μM retinol, with or without exogenous acyl CoA. The concentration of MgCl₂, when present, was 6 μM. Incubation, extraction, and analysis procedures were the same as described under Table 1. Values are means of duplicate trials, agreeing within 5%.

Lauroyl, tridecanoyl, myristoyl, palmitoyl, and stearoyl CoA, each present in the reaction mixture at 4 μM.

"ND" = "not detectable".

Corresponding acyl CoA was not included in exogenous mixture of acyl—CoA substrates.

enzymes can be extended to the unsaturated thioesters also because \(\text{Mg}^{2+}\) did not change the relative formation rate of retinyl palmitate even though palmitoyl CoA is a much better substrate for the hydrolases than other thioesters, including...
oleoyl CoA (148,149), and 2) Mg$^{2+}$ would have increased the relative formation rate of retinyl linoleate in the absence of exogenous substrate if the hydrolases had been competing with ARAT for linoleoyl CoA.

Like the microsomal thioesterases under the conditions of these specificity experiments, BSA in the reaction mixture did not interfere with interpretation of the results. Changing the concentration of BSA at a fixed concentration of exogenous acyl CoA was accompanied by small, but physiologically

<table>
<thead>
<tr>
<th>Retinyl ester</th>
<th>30 µM acyl CoA</th>
<th>60 µM acyl CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-BSA</td>
<td>+BSA</td>
</tr>
<tr>
<td>Laurate</td>
<td>5.3</td>
<td>6.0</td>
</tr>
<tr>
<td>Tridecanoate</td>
<td>9.3</td>
<td>9.9</td>
</tr>
<tr>
<td>Myristate</td>
<td>11.0</td>
<td>11.5</td>
</tr>
<tr>
<td>Palmitate</td>
<td>24.5</td>
<td>22.6</td>
</tr>
<tr>
<td>Stearate</td>
<td>24.0</td>
<td>22.9</td>
</tr>
<tr>
<td>Oleate</td>
<td>24.8</td>
<td>26.3</td>
</tr>
<tr>
<td>Linoleate</td>
<td>1.1</td>
<td>0.8</td>
</tr>
</tbody>
</table>

$^a$In potassium phosphate buffer, pH 7.4, at a final volume of 0.5 mL, rat-liver microsomes (150 µg protein) were incubated with 5 mM dithiothreitol, 52 µM retinol, and exogenous acyl CoA. The concentration of BSA, when present, was 20 µM. Lauroyl, tridecanoyl, myristoyl, palmitoyl, stearoyl, oleoyl, and linoleoyl CoA were present together in the reaction mixtures at equimolar concentrations. Values are means of duplicate trials, agreeing within 5%.
insignificant, changes in the relative reaction rate for a
given acyl-CoA derivative (Table 3).

The fatty-acid composition of retinyl esters \textit{in vivo} is
not reflected in the specificity of ARAT for its fatty-acyl-
CoA substrate \textit{in vitro}. Whereas the specificity of ARAT fails
to account for the predominance of retinyl palmitate among
esters of vitamin A in rat liver, it does accord with the
relatively small amount of polyunsaturated esters (Table 1).
The possible explanations for this discrepancy between
specificity and composition are tied to the physiological
implications, ultimately raising questions about the role of
ARAT in the synthesis of retinyl esters in the liver.

Retinyl ester hydrolase (REH; \textit{EC} 3.1.1.21) is the enzyme
that hydrolyzes retinyl esters to mobilize retinol from liver
reserves (150-153). In hydrolyzing retinyl palmitate more
slowly than other esters of retinol, this enzyme would offer
an alternate explanation for the predominance of retinyl
palmitate \textit{in vivo}. However, Mahadevan \textit{et al.} (150) found that
retinyl palmitate was preferred by REH from rat liver, the
rate being at least twice that for any other ester. Moreover,
Cooper and Olson (153), studying REH in pig liver, reported an
intermediate rate of hydrolysis for retinyl palmitate:
linolenate > myristate > palmitate > oleate > linoleate >
stearate. Thus, inasmuch as REH does not discriminate against
retinyl palmitate as a substrate \textit{in vitro}, invoking the
specificity of this enzyme to explain the predominance of retinyl palmitate in vivo is not helpful. Furthermore, when the specificities of ARAT and REH in vitro are juxtaposed, retinyl palmitate is expected to be among the least abundant retinyl esters in vivo.

An alternative to enzyme specificity as an explanation for the fatty-acid composition of retinyl esters is the possibility that palmitoyl CoA occurs in greater concentration at the intracellular site of retinol esterification than do other CoA thioesters. In partial support of this hypothesis, the data in Table 1 and Fig. 1 strongly suggest that fatty-acyl-CoA thioesters in microsomes are those of palmitic, stearic, and oleic acids; the retinyl esters formed by rat-liver microsomes in the absence of exogenous acyl CoA were palmitate, stearate, and oleate, as mentioned before.

After Drevon et al. (68) incubated rat-liver cells with retinol in culture, retinyl palmitate accounted for about 75% of the retinyl-ester fraction, whereas the stearate and oleate esters were only 10% and 4%, respectively. The contribution of retinyl oleate, however, rose to 13% when oleic acid was added to the culture medium. Ross et al. (154) found that the pattern of fatty acids in the retinyl-ester fraction of rat milk reflected the fatty-acid composition of the diet. Even trans fatty acids appeared in the retinyl esters of milk from rats fed hydrogenated corn oil.
In whole-animal experiments, Furr et al. (93) recently demonstrated the influence of the composition of dietary fatty acids on the composition of retinyl esters \textit{in vivo}. Rats were fed a diet 8% in a synthetic simple triacylglycerol or corn or partially-hydrogenated soybean oil for 23 days. Although retinyl palmitate remained dominant, the proportion of retinyl stearate or oleate increased when rats were fed tristearin or triolein, respectively. Whereas feeding rats trilinolein increased the proportion of retinyl linoleate, it decreased the total concentration of retinyl esters in the liver, consistent with the observation that CoA thioesters of polyunsaturated fatty acids are poor substrates for ARAT (Table 1). Likewise, feeding rats corn or partially-hydrogenated soybean oil enriched the retinyl esters in polyunsaturated fatty acids.

These studies have firmly established a link between the supply of fatty acids to the cell and the composition of retinyl esters. Thus, although several CoA thioesters are effective substrates for ARAT, the synthesis of retinyl palmitate \textit{in vivo} is favored perhaps by the presence of palmitoyl CoA at a concentration higher than that of other derivatives in the microenvironment of ARAT. \textit{De-novo} fatty-acid biosynthesis, along with subsequent elongation and desaturation by enzymes in the endoplasmic reticulum, contribute to the liver's pool of fatty acids. It is well-
known that the major products of these processes in mammals are palmitic, stearic, and oleic acids, the three most abundant ones in the retinyl-ester fraction of the liver (Table 1; Part I, p. 26, Table 2).

Inconsistency between the specificity of ARAT and the fatty-acid composition of retinyl esters was seen also for polar-bear liver (Table 4). Although the experimental approach differed slightly from that used for rat liver (see "General Protocol", pp. 70-73), the specificities of hepatic ARAT from the rat and the polar bear were very similar, except that the bear enzyme displayed a greater preference for oleoyl CoA than did the rat enzyme. The proportion of retinyl oleate and linoleate were higher in the bear liver than in rat liver, although retinyl palmitate predominated in both (Tables 1 and 4). In light of the results of Furr et al. (93), the fatty acids of the smelt in this bear's diet may have had an impact on the composition of retinyl esters in the liver; the fact that fish oil is rich in unsaturated fatty acids is common knowledge.

Discrepancies between specificity in vitro and composition in vivo are not unique to ARAT. An example of another acyltransferase for which such a discrepancy exists is ACAT, the analogous enzyme in cholesterol esterification. For this enzyme, oleoyl, palmitoyl, and stearoyl CoA are effective substrates, while linoleoyl CoA is less so (67). However, in
Table 4. Comparison of formation rates for retinyl esters synthesized in vitro by polar–bear–liver ARAT with their concentrations in vivo

<table>
<thead>
<tr>
<th>Retinyl ester</th>
<th>Percentage of total rate&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percentage of total hepatic retinyl-ester concentration&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>saturated acyl CoA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>saturated + unsaturated acyl CoA&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Laurate</td>
<td>4.6</td>
<td>NI&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tridecanoate</td>
<td>7.2</td>
<td>5.4</td>
</tr>
<tr>
<td>Myristate</td>
<td>13.5</td>
<td>NI</td>
</tr>
<tr>
<td>Pentadecanoate</td>
<td>11.4</td>
<td>9.0</td>
</tr>
<tr>
<td>Palmitate</td>
<td>14.5</td>
<td>18.5</td>
</tr>
<tr>
<td>Heptadecanoate</td>
<td>13.3</td>
<td>8.6</td>
</tr>
<tr>
<td>Stearate</td>
<td>9.5</td>
<td>9.7</td>
</tr>
<tr>
<td>Nonadecanoate</td>
<td>10.7</td>
<td>NI</td>
</tr>
<tr>
<td>Arachidate</td>
<td>10.4</td>
<td>8.2</td>
</tr>
<tr>
<td>Behenate</td>
<td>5.0</td>
<td>4.1</td>
</tr>
<tr>
<td>Oleate</td>
<td>NI</td>
<td>31.7</td>
</tr>
<tr>
<td>Linoleate</td>
<td>NI</td>
<td>4.6</td>
</tr>
<tr>
<td>Linolenate</td>
<td>NI</td>
<td>0.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>Reaction mixtures comprised polar–bear–liver microsomes (315 μg protein/mL), 15 μM BSA, 5 mM dithiothreitol, 80 μM retinol, and fatty–acyl–CoA thioesters in 150 mM potassium phosphate buffer, pH 7.4. After incubation at 37°C, reaction was stopped with ethanol, the mixture was extracted with hexane, and the extract was analyzed by reversed–phase HPLC. Values are means of duplicate trials, agreeing within 5%.

<sup>b</sup>The 13 corresponding saturated exogenous fatty–acyl–CoA thioesters were included in the reaction mixture, each at 10 μM. See "General Protocol" for details.

<sup>c</sup>The 10 corresponding fatty–acyl–CoA thioesters, saturated and unsaturated, were included in the reaction mixture, each at 10 μM. See "General Protocol" for details.

<sup>d</sup>Values in Table 2, Part I, p. 26, are reproduced here.

<sup>e</sup>"NI" = corresponding acyl CoA "not included" in mixture.

<sup>f</sup>"NR" = "not reported".
rat-liver homogenates cholesteryl oleate and linoleate predominate, together accounting for nearly 60% of the total cholesteryl esters (67).

Another example is acyl CoA:estradiol 17-β-acyltransferase (EC 2.3.1...), from bovine placental cotyledon. Whereas palmitoyl CoA is an effective substrate for this enzyme, estradiol in vivo is esterified mainly with polyunsaturated fatty acids (155). Like ARAT, both this enzyme and ACAT are membrane-bound also, and the effective substrate concentration in their microenvironments may likewise influence the fatty-acid composition of the lipids they acylate in vivo.

Another explanation for the discrepancy between the specificity of ARAT in vitro and the fatty-acid composition of retinyl esters in vivo is the existence of a second retinol-acylating enzyme in the cell. Although ARAT has been characterized in the small intestine (55,56), an acyl-CoA-independent enzyme that esterifies retinol has been identified in small-intestinal microsomes by Ong et al. (15). When these microsomes were incubated with retinol bound to CRBP(II), the retinyl esters formed, in order of descending percentage of the total formed, were palmitate, stearate, oleate, and linoleate. As mentioned before (Part I, p. 36), whereas little fatty-acid-specificity was seen for ARAT from small intestine (55,56), the ester proportioning observed with the
acyl-CoA-independent reaction was very similar to that found in chylomicrons (156) and whole lymph (157).

Futterman and Andrews (2) published the only data in the literature hinting at the existence of another retinol-acylating enzyme in the liver. When cat-liver microsomes were incubated with retinol, two pH optima were seen, one about 4.5 and the other about 8.2. At the latter pH the composition of retinyl esters synthesized \textit{in vitro} resembled that of retinyl esters \textit{in vivo}. Although Ross (1) did not report precisely how ARAT activity \textit{in vitro} varied with pH, the pH of all reaction mixtures in those experiments, and in the present study, was 7.4. Furthermore, Futterman and Andrews (2), like Ong et al. (15), saw no requirement for fatty acids activated with CoA. In the light of Futterman and Andrews' (2) report, the data on the specificity of ARAT \textit{in vitro} (Tables 1 and 4, Fig. 1) call for investigation into the possible existence of a second retinol-acylating enzyme in the liver.
GENERAL DISCUSSION

The research described in the preceding three sections addresses certain questions surrounding the ARAT-catalyzed transacylation of retinol that have been raised, but left unattended, by the biochemical literature. The fundamental novel findings in this work are 1) that ARAT is present in polar-bear liver and that some of its properties are different from those of rat-liver ARAT, 2) that the fatty-acid composition of retinyl esters in polar-bear liver is similar to that in other species, 3) that rat mammary tumor also apparently contains ARAT activity, 4) that ARAT activity in both this tumor and rat liver is enhanced in response to administration of vitamin A to the animal, 5) that various retinoids, including 13-c-RA, can inhibit ARAT \textit{in vitro}, 6) that 13-c-RA also inhibits benzo(a)pyrene hydroxylation and disrupts microsomal membranes \textit{in vitro}, 7) that MeROL is not an effective substrate for ARAT \textit{in vitro}, 8) that saturated C_{12-20} fatty-acyl-CoA thioesters, even- or odd-chain, are effective substrates for rat-liver ARAT \textit{in vitro} whereas polyunsaturated derivatives are not, and 9) that the acyl-CoA specificity of ARAT from rat and polar bear are similar.

With characterization of ARAT in polar-bear liver, a marine mammal joins the growing list of species in which the enzyme has been identified: other mammals, including the human
(53,56), rat (1,55,58,59), lamb (57), and mouse (60), and at
least one non-mammal, the chick (54). Thus, ARAT may be
ubiquitous in the animal kingdom. Furthermore, rat mammary
tumor joins the growing list of tissues with ARAT activity:
the liver (1,53,56), small intestine (55,56), kidney (57),
mamma (58), testis (59), and epidermis (60). Assigning a
physiological role to the ARAT-catalyzed reaction is justified
by 1) the high activity of ARAT in polar-bear liver (Part I,
p. 27), a tissue with unusually large amounts of retinyl ester
(74,75; Part I, p. 25), 2) its low activity in rat mammary
tumor, a tissue with negligible amounts (88; O. Amedee-Manesme
and H. C. Furr, Dept. of Biochemistry and Biophysics, Iowa
State University, Ames, Iowa, personal communication), 3) its
enhancement in tumor (Part I, pp. 35-36) and in liver (72,73;
Part I, pp. 35-36) by dosing with vitamin A, and 4) its
inability to use MeROL (Part II, p. 61) or polyunsaturated
acyl-CoA thioesters as substrates in vitro (Part III, p. 86),
in that MeROL is not esterified or stored in the liver (117)
and polyunsaturated retinyl esters occur only in small amounts
(Part III, pp. 76-77, Table 1). This is in keeping with the
report of Blomhoff et al. (158), demonstrating ARAT at very
high specific activity in the stellate cells of the liver, the
cells in which 80-90% of hepatic vitamin A is stored (159).
Olson (160) has proposed that ARAT has a "key role" in retinol
esterification in vivo.
The physiological relevance of the inhibition of ARAT in vitro by various retinoids (60,62,77; Part II, pp. 52,61) depends on the specificity of the in-vitro effects of the inhibitor. For example, although 13-C-RA inhibited ARAT in vitro, it did so only at physiologically unreasonable concentrations (Part II, p. 57), and not without inhibiting benzo(a)pyrene hydroxylation also (Part II, p. 60), as well as rendering the microsomes "leaky" (Part II, p. 48).

The molecular specificity displayed by 13-C-RA in disrupting membranes was striking (Part II, p. 48). Even all-t-RA, which inhibited ARAT to about the same extent as 13-C-RA (Part II, pp. 54,56), had much less effect on the integrity of the microsomes in vitro (Part II, p. 48). In the retinoic acids, then, are found two retinoids that inhibit ARAT in vitro, one increasing membrane permeability (13-C-RA) and the other not (all-t-RA). The possibility that 13-C-RA, as a drug, also disrupts membranes in vivo should now be explored.

One explanation for the discrepancy between the fatty-acid composition of retinyl esters in vivo and the specificity of ARAT for its acyl-CoA substrate in vitro is a localized high concentration of palmitoyl CoA in the microenvironment of ARAT in the endoplasmic reticulum (Part III, p. 90). An experiment for testing this hypothesis should consider the possibility that ARAT is somehow linked to fatty-acid biosynthesis, perhaps simply by being in close proximity to the microsomal
enzymes that ligate to CoA the palmitate newly synthesized by fatty-acid synthase.

A $^{14}$C-labelled fatty acid used less than others for retinyl-ester synthesis *in vivo*, but one that can be elongated to palmitate by fatty-acid synthase, e.g., lauric, may be injected intravenously into nutritionally normal rats. After, say, two weeks, the livers are removed. Hepatic retinyl esters are separated by chain-length, and the peaks are collected and counted. By examining the distribution of label in the fatty acids of the retinyl-ester fraction, the importance of fatty-acid biosynthesis to retinol esterification is determined. If label is found only in retinyl palmitate, stearate, and oleate, the conclusion would be that palmitic acid was synthesized, elongated, and desaturated before retinol was esterified. However, finding most of the label in retinyl laurate, or finding a roughly equal proportioning between that ester and longer ones, would imply that ARAT does not depend on fatty-acid biosynthesis for a supply of substrate. This conclusion, moreover, would argue for the possible existence of a second retinol-acylating enzyme in the liver. Indeed, an acyl-CoA-independent retinol-acylating enzyme, whose characteristics are similar to those of the analogous enzyme in small intestine (15), has recently been discovered (D. E. Ong, P. N. MacDonald, and A. M.
In the search for a second enzyme, retinol bound to hepatic cellular retinol-binding protein may be incubated with rat-liver microsomes, as Ong et al. (15) did for small intestine. If retinol is esterified under these conditions, if the reaction is not accelerated by the addition of acyl CoA, and if the fatty-acid composition of the retinyl esters formed reflects that of retinyl esters stored in vivo, microsomal lipids should be extracted on a large scale and then fractionated by thin-layer chromatography. After zones are scraped off the plate into an organic solvent, the fractions are saponified, the fatty acids are methylated, and the methyl esters formed are analyzed by gas-liquid chromatography. Fractions with a fatty-acid profile similar to that of the retinyl esters formed are the first to be considered putative substrates in the acyl-CoA-independent esterification of retinol. This approach, of course, may entail elucidating the positional fatty-acid composition of each lipid fraction, inasmuch as the enzyme may be restricted to the fatty acids at a particular position on the glycerol backbone.
1. The transacylation of retinol by ARAT in vivo is physiologically significant. This conclusion is based on the observations that 1) ARAT activity is unusually high in polar-bear liver, a tissue that stores retinyl esters in exceptionally large amounts, 2) ARAT activity is very low in rat mammary tumor, a tissue that contains virtually no vitamin A, either as retinyl esters or retinol, 3) ARAT activity is enhanced in both tumor and liver in response to dosing with vitamin A, and 4) 15-methylretinol, which is not esterified or stored in the liver, is not an effective substrate for ARAT in vitro.

2. Some properties of ARAT from polar-bear liver differ markedly from those of rat-liver ARAT. Whereas both BSA and dithiothreitol markedly stimulated rat-liver ARAT in vitro, bear-liver ARAT was almost independent of these incubation supplements.

3. Although ARAT is inhibited in vitro by various retinoids, studies in which retinoids are evaluated as in-vitro inhibitors of ARAT should also examine the effects of these compounds on other microsomal enzymes or on the integrity of the membranes themselves. 13-Cis-Retinoic acid, which inhibited ARAT in vitro, not only inhibited benzo(a)pyrene hydroxylation also, but increased the
permeability of the microsomal membranes to mannose–6-phosphate, as evidenced by a rise in the activity of mannose–6-phosphatase. The inhibition of benzo(a)pyrene hydroxylation \textit{in vitro} by 13-cis-retinoic acid, because it occurred at lower concentrations, may be more physiologically significant than the inhibition of retinol esterification.

4. The side-effects of 13-cis-retinoic acid, which is a drug used at high doses in the treatment of recalcitrant acne, may be mediated \textit{in vivo} by membrane disruption. 13-cis-Retinoic acid increased the permeability of microsomes to mannose–6-phosphate at concentrations where four other amphiphiles were much less effective: palmitate, 3-[\textit{cholamidopropyl}]-dimethyl-ammonio]-1-propane sulfonate, all-trans-retinoic acid, and taurocholate.

5. Retinol esterification in the liver may be catalyzed by more than one enzyme. The specificity of ARAT for its fatty-acyl-CoA substrate \textit{in vitro} fails to account for the fatty-acid composition of retinyl esters \textit{in vivo}. Whereas oleoyl CoA and CoA thioesters of C_{12}–C_{20} saturated fatty acids are effective substrates for ARAT, retinol exists in the liver mainly as the palmitate ester, with lesser amounts of retinyl stearate and oleate.

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