Subcellular localization of retinoids, retinoid-binding proteins, and acyl-CoA:retinol acyltransferase in rat liver

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Abstract Studies were conducted to define the subcellular localization of endogenous retinoids (vitamin A), retinoid-binding proteins, and acyl-CoA:retinol acyltransferase (ARAT) in liver and to determine whether their distributions were affected by hepatic vitamin A content. Quantitative subcellular fractionation techniques were used. Rats were fed purified diets either containing or lacking vitamin A to obtain animals with total retinoid stores ranging from 0.5 to 172 μg of retinol equivalent per gram of liver. Liver homogenates were fractionated by differential centrifugation to yield nuclear (N), mitochondrial-lysosomal (ML), microsomal (P), and high-speed supernatant (S) fractions. N, ML, and P were washed two more times by resuspension and centrifugation to remove constituents bound nonspecifically. S was further resolved into "floating lipid" and underlying "cytosol" by prolonged ultracentrifugation. The distributions of marker constituents were not affected by vitamin A status. Most of the retinyl ester in the liver was recovered in the S fraction where it was entirely (>95%) associated with floating lipid. About half of the total free retinol was also recovered in the S fraction, but it was mostly (2/3) associated with cytosol per se. A substantial portion (30%) of the free retinol was recovered in the 3 × -washed microsomal (P) fraction. Sufficient binding capacity for retinol was present in both P (as retinol-binding protein) and S (as cellular retinol-binding protein) to quantitatively account for the amounts of free retinol present in the two fractions. ARAT activity in the liver was distributed among the subcellular fractions in a manner identical with an endoplasmic reticulum marker enzyme (NADPH-cytochrome C reductase). Neither its absolute activity nor its relative distribution among subcellular fractions was affected by the level of total vitamin A stored in the liver.—**Harrison, E. H., W. S. Blaner, D. S. Goodman, and A. C. Ross.** Subcellular localization of retinoids, retinoid-binding proteins, and acyl-CoA:retinol acyltransferase in rat liver. *J. Lipid Res.* 1987. **28:** 973–981.

Supplementary key words vitamin A • retinol • retinyl esters • differential centrifugation • subcellular fractionation

The liver is the major storage site for retinoids in the body. Under conditions of adequate vitamin A nutriture more than 95% of the retinoids found in liver are present as long chain retinyl esters, and it is generally agreed that most of the retinyl ester is found in association with lipid droplets within various types of liver cells. Nonetheless a fully quantitative evaluation of the subcellular localization of retinoids in liver is not available. In particular, only limited information is available on the extent to which retinol and its esters may be associated with other specific subcellular organelles or membrane fractions, or on the distribution of liver retinoids in vitamin A-depleted animals.

We have approached this question using analytical differential centrifugation of rat liver homogenates, followed by lipid extraction and chromatographic analysis. The purity and composition of the subcellular fraction was assessed by marker enzyme analysis. Similar approaches have been used successfully in the past to localize other polyisoprenoid lipids in liver, namely cholesterol (1) and dolichol and its esters (2).

In the early 1950s, Collins (3) and Powell and Krause (4) found about 20% of the total hepatic retinol plus retinyl esters in the mitochondrial fraction and most of the remainder in the postmitochondrial supernatant. Krinsky and Ganguly (5) extended these initial observations by studying the distribution of retinyl ester and free retinol separately among the four subcellular fractions of livers obtained from five rats with substantial stores of liver vitamin A (100–600 μg/g). Additionally, they separated the cytosol fraction into a lipid-rich, floating layer (termed "cream") and the cytosol per se. Most of the retinyl ester was recovered in the floating "cream" with only traces (10%) in all other fractions. The distribution of free

Abbreviations: ARAT, acyl-CoA:retinol acyltransferase; RBP, retinol-binding protein; CRBP, cellular retinol-binding protein; HPLC, high performance liquid chromatography; N, nuclear fraction; ML, mitochondrial-lysosomal fraction; P, microsomal fraction; S, post-microsomal supernatant fraction.

retinol was different; although about half was recovered in the "cream", about 20% was recovered in the washed microsomal membranes and 20% was in the cytosol per se. Later work by the same group (6) demonstrated a similar distribution in chicken liver homogenates. More recently, Berman, Segal, and Feeney (7) reported finding significant amounts of both retinyl ester and retinol in several crude membrane fractions isolated from liver homogenates by differential centrifugation. In none of these studies were livers of animals depleted of vitamin A stores compared to normal, vitamin A-adequate animals.

It is difficult to critically compare and evaluate these various studies on retinoid localization for a number of reasons. First, in most cases no information was provided on the distributions of any marker constituents for various subcellular organelles and thus it is not possible to assess the cytological composition of the fractions studied. Second, in some cases inadequate information was given on the recovery of the retinoids and thus on the adequacy of the fractionation and analytical methods used. Third, it is not clear to what extent hydrolysis of retinyl esters may have occurred during preparation of the fractions.

In the present study we specifically chose to employ quantitative analytical subcellular fractionation using differential centrifugation so that we could obtain an overall view of potential retinoid pools and relate our results to those obtained previously (1–7). The studies were conducted with rats having hepatic stores of total retinol ranging from 0.5 to 172 μg/g. We have also determined the levels and distributions of the plasma and intracellular binding proteins for retinol (RBP and CRBP) and have correlated these data with the levels of free retinol present in the various fractions. Finally, we present quantitative data on the subcellular distribution of the enzyme that catalyzes the synthesis of retinyl esters in liver (acyl-CoA:retinol acyltransferase, ARAT).

EXPERIMENTAL PROCEDURES

Animals and dietary treatment

Groups of male, weanling Sprague-Dawley rats were obtained from Harlan Industries, Indianapolis, IN. All rats were fed completely purified diets prepared as described previously (8). Rats were progressively depleted of liver stores of retinol by feeding them this basal diet devoid of vitamin A for periods of up to 54 days. Following depletion some rats were repleted by feeding them the basal diet supplemented with 6 μg of retinol equivalents (in the form of retinyl esters) per g of diet. Control rats were fed the diet supplemented with vitamin A from weaning.

Preparation of subcellular fractions

Liver homogenates were prepared and fractionated by differential centrifugation essentially according to the method of deDuve et al. (9) as modified by Amar-Costesec et al. (1). Rats were killed under ether anesthesia by cardiac exsanguination. A portion of the blood so obtained was used for the preparation of serum that was frozen at −20°C prior to analysis for vitamin A. All subsequent operations were carried out in subdued light and at 4°C. The liver was removed, perfused with 50 ml of 0.25 M sucrose, minced, and forced through a tissue press. Three volumes of 0.25 M sucrose were added to the tissue and the suspension was homogenized with one stroke of a motor-driven Teflon pestle in a Potter-Elvehjem homogenizer. The resulting homogenate was centrifuged successively to yield the nuclear or N fraction (1000 rpm, for 10 min in the Sorvall SS-34 rotor), the mitochondrial-lysosomal or ML fraction (12,000 rpm for 13 min in the Sorvall SS-34 rotor), the microsomal or P fraction (50,000 rpm for 30 min in the Beckman Ti 75 rotor), and the postmicrosomal supernatant or S fraction. Each particulate fraction (N, ML, and P) was washed two more times by resuspension and centrifugation under the original conditions and the combined supernatants were used for the preparation of the subsequent fraction. At each step, particular care was taken to remove floating lipid along with the supernatant. In preparing the final S fraction, the floating lipid was mixed with the clear infranatant. Aliquots of the original homogenate and all of the fractions were used immediately after preparation for the analysis of retinoids and of several marker enzymes. The marker enzymes used were cytochrome oxidase (mitochondria), N-acetyl-β-glucosaminidase (lysosomes), catalase (peroxisomes), NADPH-cytochrome C reductase (endoplasmic reticulum), alkaline phosphodiesterase (plasma membrane), and galactosyltransferase (Golgi apparatus). Additional aliquots were frozen at −80°C for subsequent analyses for protein, DNA, for the plasma and intracellular retinol-binding proteins (RBP and CRBP, respectively), and for ARAT.

Retinoid analyses

Immediately following preparation, aliquots (0.5 ml) of homogenates or subcellular fractions were pipetted into screw-capped test tubes containing 2 ml of absolute ethanol. The contents were mixed and allowed to sit at 4°C for at least 30 min. Neutral retinoids were partitioned into 15 ml of hexane (three 5-ml extractions). A number of pilot experiments demonstrated that this procedure led to the quantitative recovery of retinol and retinyl esters present in the samples. The bulk of the hexane extract was taken to dryness under a stream of N_2 and redissolved in a small volume (approximately 100–200 μl) of ethanol containing a known amount of retinyl acetate as an internal standard. A portion of this sample (20 μl) was analyzed by reverse phase HPLC on a Beckman ODS column (4.6 × 150 mm) using an isocratic mobile phase of 100% methanol at a flow rate of 2.0 ml/min. Absorbance at 326 nm was monitored with a fixed wavelength spec-

trophotometer (Beckman Model 160). Unesterified retinol was quantitated by comparison of the retinol peak with that of the retinyl acetate internal standard using a peak height ratio method. For determination of the total amount of neutral retinoid (unesterified retinol plus retinyl esters) in the sample, a small aliquot of the original hexane extract was taken to dryness, saponified, and subjected to HPLC analysis as described above. Total retinyl esters were then determined by difference.

ARAT assay

ARAT activity was assayed under conditions previously described to give maximum retinyl ester synthesis (10). Reaction tubes contained 120 nmol/ml of [^{3}H]retinol, 100 μM palmitoyl-CoA, 5 mM dithiothreitol, and 20 μM bovine serum albumin. Each tissue fraction was assayed at two protein concentrations to assure that retinol esterification was directly proportional to the amount of enzyme protein. Incubation time was 5 min.

Other biochemical analyses

Protein was assayed by the method of Lowry et al. (11) using bovine serum albumin as a standard. DNA was assayed by the fluorometric, dye-binding method of LaBarca an Paigen (12) using calf thymus DNA as a standard. RBP and CRBP were assayed using sensitive and specific radioimmunoassays for the rat proteins as previously described (8, 13–15). The following marker enzymes were assayed by the indicated published procedures: cytochrome oxidase (16), N-acetyl-β-glucosaminidase (17), catalase (18), NADPH-cytochrome C reductase (19), alkaline phosphodiesterase (20), and galactosyltransferase (21). All marker enzyme assays were conducted under conditions where product formation was proportional to the quantity of protein (enzyme) in the incubation mixture; in all cases at least two protein concentrations were used for each determination. Thus, our activity measurements are directly proportional to the amount of enzyme protein contained in each preparation.

RESULTS

Retinoid-related parameters in the rats studied

Fig. 1 shows the relationships of several retinoid-related parameters in whole homogenates to the quantity of total retinol stored in the liver. The twelve rats had hepatic total retinoid levels ranging from 0.5 to 172 μg of retinol equivalent per g. As expected, in rats with larger total stores, almost all of the liver retinoid was present as long-chain retinyl ester. As the liver was progressively depleted of retinoids, a greater relative amount was present as unesterified retinol. Indeed, as shown in Fig. 1, the hepatic content of unesterified retinold did not fall until the total retinoid content was less than about 30 μg/g. Note, however, that even in the animals with the lowest liver stores the serum retinol levels were still in the low-normal range. Thus the animals used are not truly vitamin A-deficient and we will refer to them as "vitamin A-depleted." In the vitamin A-depleted rats there was a progressive increase in the total hepatic concentration of RBP as depletion progressed, as has been reported previously (8). Note also that this increase in liver RBP occurred only when total liver retinoid fell to less than about 30 μg/g. In contrast to its effects on hepatic levels of total retinol and RBP, vitamin A depletion did not markedly alter the liver levels of CRBP or ARAT.

One depleted rat shown in Fig. 1 was unusual in that, in spite of a substantial liver total retinoid store (about 24 μg/g), its serum retinol level was in the "frankly deficient" range (< 10 μg/dl). Interestingly, this rat was also unusual in having a greatly expanded pool of RBP in the liver. It appears that the animal had a defect in the secretion of RBP and hence in the mobilization of its liver retinol. The animal exhibited no gross hepatic pathology and the reason for its apparent lack of ability to mobilize liver retinol is unknown.

Distributions of retinoid-related parameters in subcellular fractions of rat liver

For each of the rats presented in Fig. 1 a complete analytical subcellular fractionation was carried out. The distributions of total protein and of several marker constituents for various subcellular organelles among the subcellular fractions are presented in **Table 1.** The distributions agree well with those previously reported by us (22) and others (1) using the same fractionation techniques. Also as previously reported (23), we found that neither the absolute activity of any of the marker enzymes nor their relative distributions were affected by the vitamin A status of the rats. These data establish the cytological composition of our subcellular fractions and indicate that the major subcellular organelles were fairly well resolved from one another and recovered in high yield in the appropriate fraction. For example, the ML fraction contained more than 70% and 60%, respectively, of the mitochondria and lysosomes as assessed by the distribution of cytochrome oxidase and N-acetyl-β-glucosaminidase. Contamination of the fraction by microsomal vesicles derived from either the endoplasmic reticulum (NADPH-cytochrome C reductase), plasma membrane (alkaline phosphodiesterase), or Golgi apparatus (galactosyltransferase) is low as indicated by the percentage of any of these activities recovered in the ML fraction (less than 10% in each case). More important to note is the composition of the P fraction which corresponds closely to the microsomal fraction that has been exhaustively characterized by Beaufay and his colleagues (1, 20, 24, 25) in terms of its cytological composition. Our P fraction is characterized by a high yield of microsomal

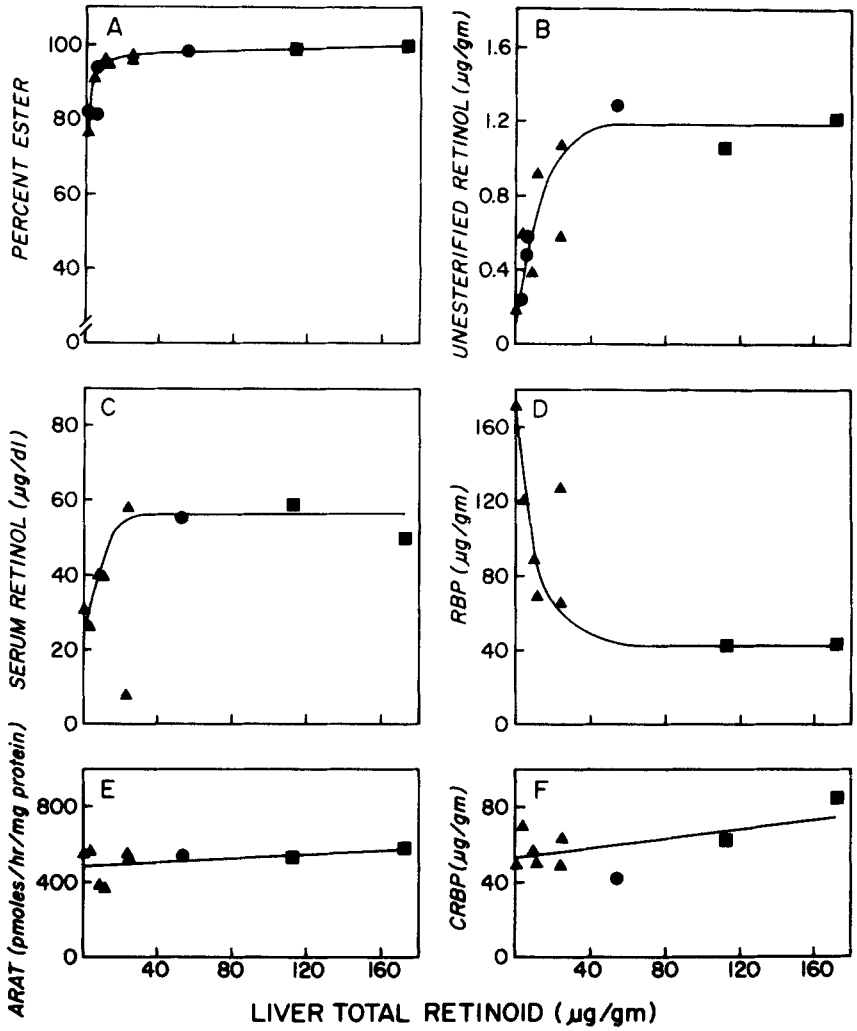

Fig. 1. Relationships of several retinoid-related parameters to the total liver retinoid store: A, percent of total hepatic neutral retinoid present as long chain retinyl ester; B, absolute content of unesterified retinol in liver; C, serum retinol level; D, liver RBP level; E, liver ARAT activity; F, liver CRBP level. Points represent individual liver homogenates. Symbols refer to control (■), vitamin A-depleted (▲), or vitamin A repleted (●) rats as defined in the text.

components such as endoplasmic reticulum (78%), plasma membrane vesicles (57%), and Golgi apparatus (75%). We estimate that only about 14% of total microsomal protein is due to contaminating mitochondria, lysosomes, and peroxisomes.[1] Applying similar calculations to the membrane-derived vesicles, Beaufay et al. (25) estimated that 7–8% of microsomal protein was contributed by plasma membrane and 4–5% was contributed by Golgi membranes. Thus about 75% [100 – (14 + 7 + 4)] of total microsomal protein is accounted for by vesicles derived from the endoplasmic reticulum per se.

The distributions of retinoids and related parameters among the fractions are presented in **Table 2** where the data for all animals are pooled. Most of the retinyl ester in the homogenates was recovered in the high speed supernatant (S) fraction. About half of the total unesterified retinol was recovered in the S fraction, but a substantial portion (30% of the total) was consistently recovered in the 3 × -washed microsome (P) fraction. Further information on the nature of the association of retinol and retinyl esters with the P and S fractions is provided below. It should be noted that although the *relative* amount of

[1]Contamination by mitochondria, lysosomes, and peroxisomes is indicated by the percentages of total recovered activity of, respectively, cytochrome oxidase (16%), N-acetyl-β-glucosaminidase (23%), and catalase (8%). These three populations of organelles have been shown to account for 20%, 0.7%, and 2.5% of total liver protein, respectively (26). From these values it is found that these organelles could contribute a maximum of about 3.6% of total homogenate protein to the microsomal fraction [i.e., (20% × 0.16) + (0.7% × 0.23) + (2.5% × 0.08)] which contains, however, 26% of total homogenate protein (Table 1).

TABLE 1. Distributions of marker enzymes in rat liver fractions

Constituent	Percent of Recovered Amount[a]				Total Recovery (%)[b]
	N	ML	P	S	
Protein (12)	14 ± 4	26 ± 4	26 ± 5	34 ± 3	94 ± 12
Cytochrome oxidase (12) (mitochondria)	13 ± 4	71 ± 11	16 ± 8	0	93 ± 18
N-Acetyl-β-glucosaminidase (11) (lysosomes)	12 ± 6	60 ± 7	23 ± 7	4 ± 1	89 ± 11
Catalase (12) (peroxisomes, in part)	9 ± 9	38 ± 12	8 ± 7	45 ± 17	115 ± 47
NADPH-cytochrome C reductase (12) (endoplasmic reticulum)	9 ± 4	8 ± 6	78 ± 1	6 ± 3	87 ± 14
Galactosyltransferase (4) (Golgi apparatus)	8 ± 2	9 ± 4	75 ± 3	7 ± 3	93 ± 10
Alkaline phosphodiesterase (9) (plasma membrane)	33 ± 5	8 ± 5	57 ± 7	2 ± 1	97 ± 15
DNA (6) (nuclei)	55 ± 3	7 ± 2	16 ± 4	22 ± 1	94 ± 15

[a]Results are given as means ± one standard deviation. The number of experiments is given in parentheses. Relative values are presented for the distribution of each constituent among the four homogenate fractions: nuclear (N), mitochondrial-lysosomal (ML), microsomal (P), and supernatant (S). The values given represent the percentage of each constituent recovered in each fraction compared to the sum of the amounts recovered in all four fractions (taken as 100%).

[b]The recoveries listed represent the total amount of a constituent recovered in all four fractions relative to the amount of that constituent in the whole homogenate.

unesterified retinol in the microsomal fraction was higher than the relative amount of retinyl esters, the *absolute* mass of retinyl esters in the P fraction exceeded the mass of retinol in all cases except at very low total liver retinoid levels (<5μg/g) **(Fig. 2).** As in whole liver (Fig. 1) the amount of unesterified retinol in both the P and S fractions (Fig. 2) was constant when total retinoid levels were greater than 20–30 μg/g. For retinyl esters the amounts in both fractions increased continuously as total liver retinoid increased.

RBP was recovered mostly in the microsomal (P) fraction whereas CRBP was almost entirely recovered in the soluble (S) fraction (Table 2). Also presented in Table 2 are quantitative fractionation data on the subcellular distribution of ARAT. The enzyme was largely recovered in the P fraction and its distribution among the fractions was identical within experimental error with that of the endoplasmic reticulum marker, NADPH-cytochrome C reductase. It was of interest to observe that the relative distributions of RBP, CRBP, ARAT, and unesterified retinol were the same regardless of the total liver store of retinoid. Only for retinyl ester was there a consistent trend; in rats with higher total stores a greater relative amount of retinyl ester was recovered in the S fraction and a smaller relative amount in the P fraction. Thus, in the seven rats with total stores below 20 μg/g, the percent of retinyl ester in S ranged from 60 to 80% and in P it ranged from 5 to 15%. In contrast, in the five rats with stores greater than 20 μg/g, the relative amount of retinyl ester in S was greater than 80% and in P it was less than 5%. These relationships between total retinoid stores and the distribution of constituents among the fractions were confirmed by regression analyses which showed no significant relationship ($P > 0.3$) for RBP, CRBP, ARAT, and unesterified retinol, but a statistically significant ($P = 0.025$) relationship for retinyl ester. The latter relationship apparently results from the finite capacity for retinyl ester to associate with microsomal membranes (Fig. 2, top) so that, as the total amount of ester present in the homogenate increases, the relative amount in P

TABLE 2. Distribution of retinoid-related parameters in rat liver fractions[a]

Fraction	Percent of Recovered Amount				Recovery
	N	ML	P	S	
Retinol (12)	10 ± 4	8 ± 4	30 ± 9	53 ± 8	98 ± 27
Retinyl esters (12)	15 ± 9	3 ± 2	7 ± 4	75 ± 10	88 ± 14
RBP (8)	8 ± 4	6 ± 3	80 ± 6	6 ± 2	90 ± 15
CRBP (9)	5 ± 1	2 ± 3	1 ± 2	92 ± 3	86 ± 12
ARAT (6)	13 ± 2	8 ± 3	72 ± 7	7 ± 5	103 ± 9

[a]Results are presented as in Table 1.

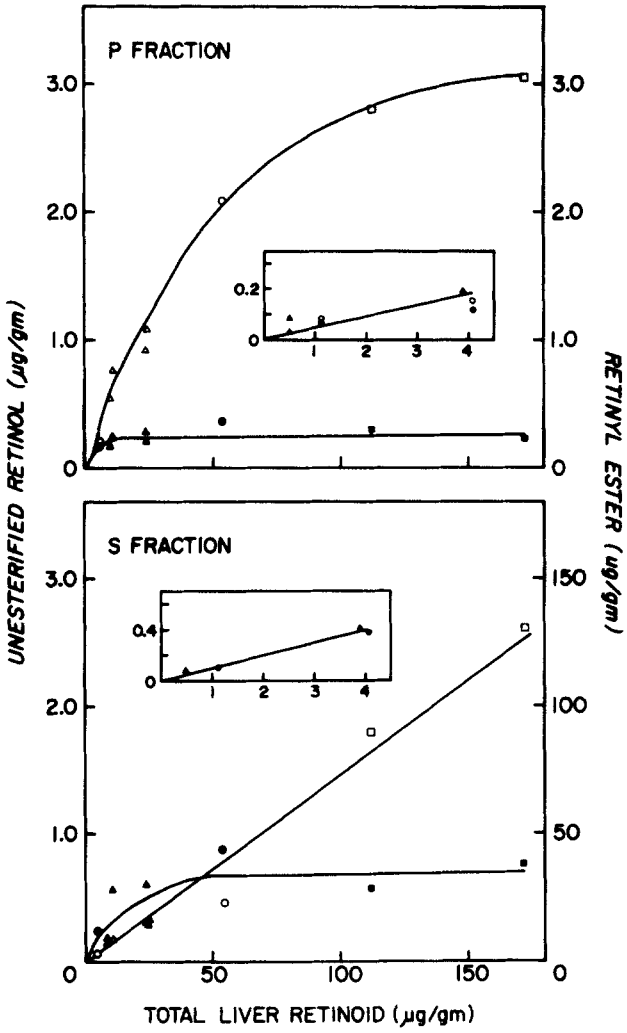

Fig. 2. Relationship of total liver retinoid store to the content of unesterified retinol (closed symbols) and of retinyl esters (open symbols) in the microsomal (P) and soluble (S) fractions of rat liver homogenates. Symbols refer to control (squares), vitamin A-depleted (triangles), or vitamin A-repleted (circles) rats as defined in the text. Insets show on an expanded scale these relationships at total liver retinoid stores of less than 5 μg/g.

decreases and a greater relative amount is then recovered in the S fraction.

Association of retinoids with the microsomal and soluble fractions

In order to further define the nature of the association of the various forms of retinol (free vs. ester) with the S fraction, the fraction was subjected to prolonged ultracentrifugation to separate "floating lipid" from the underlying "cytosol" per se. Each of these separated fractions was then analyzed for free and esterified retinol as before and the results are shown in **Table 3.** Almost all of the retinyl ester was recovered in the floating lipid layer, whereas most of the unesterified retinol was present in the underlying cytosol.

Experiments were also conducted to ask whether retinoids present in the P fraction might have arisen as a result of artefactual redistribution of retinol and retinyl esters from cytosol to the microsomal membranes during homogenization. In order to assess this possibility, a mixing experiment was conducted. A microsomal (P) fraction nearly devoid of retinoids was prepared from the liver of a vitamin A-deficient rat (having a liver total retinol of 0.05 μg/g), and a soluble (S) fraction was prepared from the liver of a vitamin A-adequate animal that had a liver total retinol level of 55 μg/g. The microsomal pellet from the deficient rat was then resuspended in a volume of S fraction from the control rat derived from an equivalent amount of tissue. This suspension was then diluted with 0.25 M sucrose to give a final homogenate concentration equivalent to our normal starting homogenates (i.e., 1 g of tissue in a final volume of 4 ml) and then homogenized and allowed to sit at 4°C for 30 min. The "homogenate" was then centrifuged to reisolate a P fraction in the usual way. We observed that 2.5% of the retinyl ester originally in the S fractions was now recovered in the washed microsomes. Of the unesterified retinol originally present in the S fraction, 9.5% was recovered in the washed microsomes. Thus, although a measurable transfer of retinoids was observed, it was much smaller than the amounts of retinol and retinyl ester routinely found associated with microsomes (i.e., about 30 and 7%, respectively).

Two experiments were conducted in which microsomes from vitamin A-adequate rats were washed with solutions of EDTA or pyrophosphate known to remove bound ribosomes and "soluble" proteins that adsorb to the microsomal vesicles (24). Microsomal pellets were resuspended by homogenization in a solution of either *a*) 0.25 M sucrose–0.05 M EDTA, pH 7.4, *b*) 0.25 M sucrose–0.015 M sodium pyrophosphate, pH 8.2, or *c*) 0.25 M sucrose alone (as a control). Washing with either of the first two solutions resulted in the removal of 25% of the microsomal protein compared with 8% for the washing with 0.25 M sucrose alone. However, EDTA or pyrophosphate treatment did *not* remove any more of the retinoid associated with the original microsome pellet than did washing with 0.25 M sucrose (<10%). Thus, these experiments provided evidence that the association of retinoids with the microsomal vesicles of the P fraction is not due to nonspecific adsorption of the compounds to the microsomal membranes.

Finally, two experiments were conducted to assess the quantity of retinoid associated with purified Golgi apparatus. Golgi membranes were isolated from the livers of rats having a total liver retinoid content of 30 and 105 μg/g, respectively, using discontinuous sucrose gradients as described by Morré (27) and used by us previously (23). The isolated Golgi membrane contained about 1.1% of total homogenate protein and 2.7% and 2.0% of the total unesterified retinol and retinyl esters, respectively.

TABLE 3. Distribution of endogenous retinoids between "floating lipid" and "cytosol" of the S fraction[a]

Constituent	Percent of Recovered Amount[b]		
	Floating Lipid	Cytosol	Total Recovery (%)[c]
Volume (3)	30 ± 3	70 ± 3	91 ± 6
Protein (1)	14	86	72
Retinol (3)	32 ± 16	68 ± 16	100 ± 14
Retinyl esters (3)	97 ± 4	3 ± 3	78 ± 21

[a] High speed supernatant (S) fractions were prepared in the usual way. The supernatants were overlayered with a small volume of water and centrifuged at 50,000 rpm for 16 hr in the Beckman Ti 75 fixed-angle rotor. The contents of the upper part of the tube (ca. 30% of total volume) were removed with a pipette and are referred to as "floating lipid." The remaining infranatant solution is referred to as "cytosol." The three individual rats used in these experiments had liver total retinoid levels of 0.3 μg/g (76.0% ester), 57.9 μg/g (98.7% ester), and 77.3 μg/g (98.6% ester).

[b] Results are given as means ± one standard deviation for the number of experiments indicated in parentheses. The values represent the percentage of the constituent in each fraction compared to the sum of the amounts in the two fractions (taken as 100%).

[c] The recoveries listed represent the total amount recovered in the "floating lipid" plus "cytosol" relative to the total amount in the unfractionated high speed supernatant (S fraction).

Thus, only a slight enrichment of retinoid was observed in the Golgi fraction.

DISCUSSION

This study reports detailed quantitative information about the distributions of retinoids and related parameters among well-defined subcellular fractions of liver. Clearly, the bulk of the retinyl ester in liver is associated with the postmicrosomal supernatant fraction and, in particular, with "floating lipid." This result is consistent with the idea that most of the retinyl ester is stored in lipid droplets within various liver cell types (and particularly within stellate cells, see below). As liver retinoid increased from low concentrations into the adequate range, both the absolute amount and percent of liver retinyl ester increased in this S fraction. The localization of unesterified retinol was quite different from that of retinyl esters. Thus, although about half of the retinol was associated with the soluble "S" fraction, it was not enriched in the "floating lipid" but rather it was present mostly in the cytosol per se. A substantial relative amount (30% of the total) was present in the washed microsomal (P) fraction. It appears that most of the microsomally associated retinol is associated with the endoplasmic reticulum per se, based on the composition of our microsome fraction, experiments with purified Golgi membranes, and previous results in the literature. The slight enrichment of retinoids observed in Golgi membranes (two- to threefold over the total homogenate) was comparable to that observed previously by Nyquist, Crane, and Morré (28) and could be entirely due to the level of contamination of the preparations with endoplasmic reticulum (23). Results similar to these have been reported by Mack et al. (29) regarding the retinoid content of purified rat liver plasma membranes. These authors found that a highly purified plasma membrane preparation containing about 1% of the homogenate protein contained only about 2% of the total homogenate vitamin A (retinoid), thus suggesting that plasma membranes are not a major locus of membrane-associated retinoids in liver.

Liver contains two proteins with high affinity and specificity for binding unesterified retinol, namely RBP that is synthesized in liver for release into plasma (30) and CRBP that is found in tissues but not plasma (31). These two proteins are almost exclusively confined to the P and S fractions of liver homogenates, respectively. Previous work demonstrated that RBP was enriched in both rough and smooth microsomal fractions (32) and that, although both plasma membranes (33) and Golgi membranes (23) contained RBP, neither membrane fraction was a major site of localization of RBP in liver. It is of interest to note that, in each of the major subcellular fractions containing unesterified retinol, a binding protein for retinol is found. When we compared the absolute levels of ligand (retinol) and binding protein in each of these cell fractions (P and S) it was found that the amount of binding protein equalled or exceeded the amount of unesterified retinol present. Thus the molar ratio of retinol:RBP in the P fraction varied from 0.05 in very depleted liver to 0.5–0.6 in control animals. Likewise, the molar ratio of retinol:CRBP in the S fraction varied from 0.1 to 0.7–0.8. Therefore, we would suggest that the unesterified retinol in the cytoplasm may be bound to CRBP and in transit to the endoplasmic reticulum for mobilization via RBP or to other sites of metabolism or action. Likewise, much of the unesterified retinol in microsomes may be complexed with RBP and destined for secretion from the liver. Alternatively, the microsomal free retinol could conceivably serve as the endogenous substrate for ARAT.

In the normal rat, retinoids are found in large amounts in both hepatic parenchymal cells and stellate (fat-storing) cells. Recent studies (34, 35) have demonstrated that the stellate cells are enriched in retinoids, in the cellular retinoid-binding proteins, and in the enzymes that synthesize (ARAT) and hydrolyze retinyl esters. For RBP, CRBP, and ARAT, the parenchymal cells contain about 98%, 91%, and 80%, respectively, of the total liver content of these retinoid-related parameters. In contrast, most (about 80%) of the retinyl ester is in stellate cells. Quantitative information on the cellular distribution of unesterified retinol was not reported (34, 35).

In the present studies, the components of the subcellular fractions obviously originate from all types of liver cells. From the work of Blouin, Bolender, and Weibel (36), the contribution that each liver cell type makes to the various organelle populations can be assessed. It was esti-

mated (36) that about 55% (by volume) of the lipid droplets in the liver are contributed by the stellate cells, with the remainder being in hepatocytes. Thus, a substantial portion of the retinyl ester we have found associated with "floating lipid" likely arises from the stellate cells, which account for only 1.4% of the total volume of the hepatic parenchyma (in contrast to hepatocytes themselves, which account for 78% of parenchymal volume). The contribution of stellate cells to microsomal membrane components and the cytoplasm per se is very small. They contribute only 1.5% of the cytoplasmic volume, while hepatocytes contribute 93%. In terms of cellular membranes, the contribution of hepatocytes and stellate cells, respectively, are: plasma membrane, 73.5% versus 7.1%; Golgi membranes, 84.9% versus 1.2%, and endoplasmic reticulum, 93.6% versus 1.1%. Thus, although the stellate cells contribute significantly to the retinoids associated with lipid droplets, their contribution to the microsomally associated unesterified retinol is probably small.

We acknowledge with thanks the excellent technical assistance of Ms. R. Stoltzfus and Ms. R. Piantedosi. This work was supported in part by NIH Grants AM-32096, DK-05968, HL-22633, HD-00691, and HD-16484.

Manuscript received 9 October 1986 and in revised form 13 February 1987.

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