Uptake and metabolism of retinol (vitamin A) in the isolated perfused rat liver

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SUMMARY The isolated perfused liver was employed to study various aspects of retinol-C^{14} metabolism. The rate of uptake of retinol, retinal, retinoic acid, and retinol acetate was rapid (first-order rate constant, $k = 0.12–0.16$ min^{-1}) during the first 3–10 min of perfusion, and then decreased quickly. After 1 hr of perfusion, 50–60% of perfusate retinal-C^{14}, 25–30% of retinol-C^{14}, and 20% of retinol-C^{14} acetate were found in the liver as retinol-C^{14} ester, and about 10% of the original perfusate radioactivity appeared in the bile as a group of water-soluble metabolites. The amount of retinol ester formed in the liver during 1 hr was linearly dependent on the concentration of retinol in the perfusate over the range of 3 to 1000 μg/100 ml. Serum, bile salt, or prior injections of India ink had no apparent effect on the formation and storage of retinol ester during perfusion. Only the water-soluble metabolites, but not retinol and its associated derivatives, were released into the perfusate and bile. After 1 hr of perfusion, about 20% of the retinoic acid-C^{14} was found in the liver as free retinoic acid, and 5% was present in a nonionic fraction.

KEY WORDS Vitamin A-C^{14} · retinol-C^{14} · retinoic acid · retinal · uptake · metabolism · rat · liver perfusion · bile · bile metabolite · serum · esterification · Tween 80

MAMMALIAN LIVER plays a central role in the storage and metabolism of the fat soluble vitamin, retinol. Orally administered retinol, after transport across the intestine and into the lymph, is taken up mainly by the liver (1), stored as its ester, and released when needed to maintain a normal concentration in the plasma (2). Within the liver, retinol may be isomerized (3) or reversibly oxidized to retinal (4–6), and retinal may be oxidized further to retinoic acid (7). Difficulty has been encountered in demonstrating the synthesis or breakdown of long-chain fatty acid esters of retinol by liver tissue, however, and the metabolic fate of retinoic acid is largely unknown (8–10).

Information concerning the role of the liver in retinol storage and metabolism has mainly been obtained by the use of intact animals, liver slices, and homogenates, procedures which are of limited value in elucidating physiological events in a single whole organ. The isolated perfused liver technique, however, allows investigation of the behavior of the organ under conditions approximating those in the intact animal, and yet permits a more careful control of experimental parameters than is possible in vivo. Worker (11) has demonstrated the feasibility of using this technique for an investigation of the uptake and esterification of retinol.

In the present investigation, several facets of retinol metabolism have been studied by use of the isolated perfused rat liver: the relative rate of uptake of various retinol componds, the rate of the formation of retinol ester, the dependence of the uptake rate on the perfusate retinol concentration, and the production and biliary excretion of metabolites of retinol-C^{14}.

EXPERIMENTAL PROCEDURES

Materials

Retinol acetate, retinol, and retinoic acid, labeled with C^{14} in the 6 and 7 positions, were kindly supplied by Dr. O. Wiss of Hoffman-LaRoche and Co., Basel. After oxidation of labeled retinol with MnO_2 (12), retinal-C^{14} was isolated by alumina chromatography. Retinol compounds were purified by alumina chromatography at least at monthly intervals and were stored at $-10°$ in low actinic glassware. Retinoic acid was purified by silicic acid chromatography.

Liver Perfusions and Analyses

Male rats (Rolfsmeyer Farm, Madison, Wis.) which weighed 200–350 g were used. Details of the perfusion apparatus, the perfusion technique employed, and the

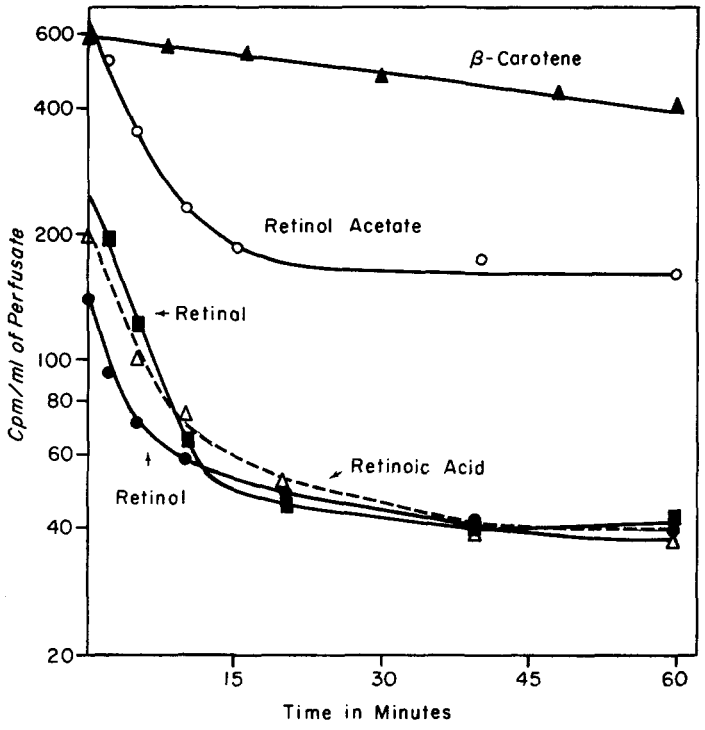

FIG. 1. Kinetics of the uptake of retinol-C^{14} compounds. Initial perfusate concentrations were: ○, retinol acetate (0.01–0.03 μg/ml); ●, retinol (0.01–0.03 μg/ml); ■, retinal (0.25 μg/ml); △, retinoic acid (0.25 μg/ml); and ▲, β-carotene (0.2 μg/ml).

method of analyzing livers for the various retinol compounds have been published elsewhere (13). In brief, the portal vein was cannulated and the liver was perfused with a solution of 0.5% glucose in Krebs-Ringer bicarbonate buffer, pH 7.4, which had been equilibrated at 38° with 95% O_2–5% CO_2. The liver was removed, placed in the perfusion apparatus, and 98 ml of the same buffer was perfused and recirculated through the liver. At zero time the radioactive substrate (retinol, retinol acetate, retinal, or retinoic acid), solubilized in 0.1 ml of Tween 80 (polyoxyethylene sorbitan monooleate) and 2 ml of buffer, was added to the perfusate. Two milliliter aliquots of the perfusate were withdrawn at various times, an equal volume of 95% ethanol was added to each aliquot, and the substrate was extracted for 8–10 min with 2 volumes of *n*-hexane. Aliquots of the hexane phase were plated as infinitely thin samples on aluminum planchets and counted in a windowless gas flow Geiger-Müller counter.

After perfusion, the livers were flushed with saline and homogenized in a Waring blendor with 100 ml of hexane–absolute ethanol 3 : 1 (v/v). The latter contained approximately 200 μg each of unlabeled retinol acetate, retinol, and retinal as carrier. After evaporating most of the solvent under reduced pressure, the lipid extract was chromatographed on water-deactivated alumina. The fractions collected with a given eluent, in the order of their elution, were: retinol ester (hexane), retinal (1–2% acetone in hexane), retinol (5% acetone in hexane), and terminal polar fractions (acetone). In some experiments, minor hepatic lobes were tied off and removed during the perfusion. The lipids in these lobes were extracted and chromatographed in a like manner. Bile was collected by cannulation of the bile duct before perfusion.

RESULTS

The Rate of Uptake of Retinol Derivatives from the Perfusate

The initial rates of removal of retinol, retinol acetate, retinal, and retinoic acid from the perfusate at low concentrations (0.01–0.25 μg/ml) were roughly comparable (Fig. 1). The initial rate continued for approximately 10 min with retinal, and for 3–8 min with the other substrates, followed by a 5- to 10-fold slower rate during later periods. In contrast, the initial rate of β-carotene uptake was much slower (13). β-Carotene (0.2–0.4 μg/ml) had no effect on retinol uptake, and conversely, retinol (0.15–5.0 μg/ml) did not affect the removal of β-carotene or retinal from the perfusate.

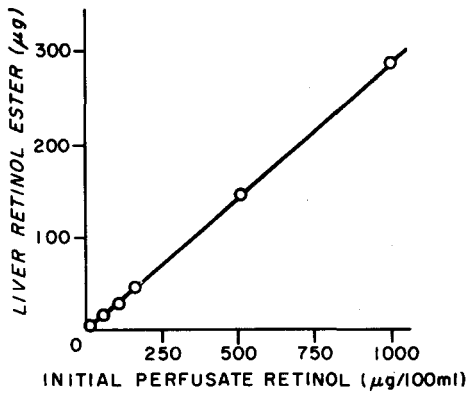

FIG. 2. Effect of the initial concentration of retinol-C^{14} in the perfusate on the amount of retinol-C^{14} ester in the liver after 1 hr of perfusion.

The Distribution of Radioactive Compounds in the Liver

After perfusion of liver for 1 hr with retinal, retinol, or retinol acetate, the major class of compounds found in liver was retinol ester. When expressed as a percentage of the substrate initially present in the perfusate, 50–60% of retinal-C^{14}, 25–30% of retinol-C^{14}, and 20% of retinol-C^{14} acetate were found as retinol-C^{14} ester (Table 1). The higher percentage of retinal found as retinol-C^{14} ester may be due to greater uptake of retinal, or possibly to differences in the pool sizes of retinal, "bound retinol," and free retinol in the liver. In addition to liver retinol ester, 2–10% of the initial perfusate radioactivity for all of these substrates was found in hexane-extractable components more polar than retinol ester. Compared to β-carotene, retinal and retinol were converted to liver retinol ester much more effectively.

When retinoic acid was employed as the substrate, about 20% of perfusate retinoic acid-C^{14} was retained in the liver as retinoic acid after 1 hr of perfusion, while 4–7% of the original radioactivity appeared in other unidentified materials which were eluted from silicic acid columns after retinoic acid. In keeping with many other studies, neither retinol nor retinol ester was found.

For all the compounds studied, the aqueous $CaCl_2$ phase, which was used to wash the hexane extract of perfused liver, contained an average of 8–10% of the original perfusate radioactivity. This material was not further characterized. The over-all recovery of radioactivity from the liver, perfusate, and bile after 1 hr of perfusion ranged from 80 to 95% of the original radioactivity added (Table 1).

Effect of the Retinol Concentration of the Perfusate on the Formation of Liver Retinol Ester

The formation of liver retinol ester was linearly dependent on the retinol concentration in the perfusate over the range from 3 to 1000 μg/100 ml of perfusate (Fig. 2). In contrast, the formation of retinol ester from β-carotene was shown to reach a plateau with concentrations of perfusate β-carotene of only 60–80 μg/100 ml of perfusate (13). Concentrations or retinol greater than 1000 μg/100 ml could not be readily achieved in 0.1% Tween 80. Since the formation of retinol ester was inhibited 15% by 0.3% Tween 80 and 60% by 1% Tween 80, the minimal adequate amount of the detergent (0.1%) was used in all experiments.

Effect of Rat Serum, Bile, or India Ink on Retinol Metabolism in Perfused Liver

Neither fasted rat serum, at concentrations of 2, 6, 12, or 22% in perfusate (v/v), nor donor rat bile, at concentrations of 1, 5, or 10% in perfusate (v/v), had any significant effect on the amount of liver retinol-C^{14} ester formed from perfusate retinol-C^{14} during 1 hr of perfusion. One day after the reticuloendothelial system

TABLE 1 PERCENTAGE OF THE INITIAL PERFUSATE RADIOACTIVITY IN VARIOUS FRACTIONS OF LIVER, PERFUSATE, AND BILE AFTER ONE HR OF PERFUSION

Substance	Initial Amount in Perfusate	In Liver: Hexane Extractable: Retinol Ester*	In Liver: Hexane Extractable: Unidentified More Polar Compounds	In Liver: Other Compounds	In Liver: $CaCl_2$ Wash Layer	In Perfusate as Original Compound	In Perfusate and Bile as Water-Soluble Compounds	Average Total Recovery
	μg		%				%	%
Retinal	25	57 ± 4.6	5–10	—	8	20–25	5–10	95
Retinol	1	26 ± 2.9	2–7	—	11	30–35	10–15	85
Retinol acetate	1	19 ± 2.7	4–6	—	—	30–35	—	—
Retinoic acid	25	—	4–7	19 ± 2.7†	14	35	5–15	85
β-Carotene‡	20	4.9 ± 0.4	—	5–6§	5	60–65	5	82

* Average of 3 to 6 perfusions.
† Liver retinoic acid-C^{14}.
‡ β-Carotene data taken from reference 13.
§ Liver β-carotene.

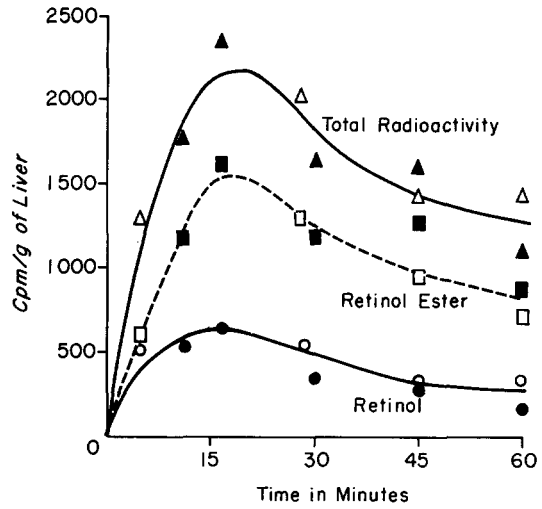

FIG. 3. Effect of perfusion time on the amount of radioactivity in the liver. At various times, lobes of a liver being perfused with retinol-C^{14} were tied off, removed, and analyzed for total radioactivity, retinol-C^{14} ester and retinol-C^{14} (△, □, ○). In other experiments, whole livers were perfused for various times and analyzed similarly (▲, ■, ●). Open symbols denote experiments with tied lobes, solid symbols those with whole livers.

of rats was challenged by an intraperitoneal injection of 1 ml of a 20% solution of India ink in saline, the uptake, esterification, and storage of retinol-C^{14} by perfused livers of these animals was normal.

Effect of Length of Perfusion

The effect of perfusion time on the distribution of radioactive compounds in liver was studied by removing lobes from a perfusing liver at various times and by chromatographing the hexane extract of these tissue samples on alumina. The total radioactivity in the tissue reached a maximum within 20–30 min and then declined (Fig. 3). Analysis of whole livers perfused for various times gave identical results. Of the hexane-extractable radioactivity, 90–95% was in retinol and retinol ester. The ratio of retinol-C^{14} to retinol-C^{14} ester in the liver was 1:1 in less than 10 min of perfusion, but was 1:7 after 1 hr. The 100% acetone fractions of liver extracts, representing unidentified oxidation products, contained about 10% of the liver radioactivity at all times.

Distribution of Radioactivity in Perfusates

After 1 hr of perfusion with retinol-C^{14}, 85–90% of the remaining radioactivity in the hexane extract of the perfusate was unchanged retinol. About 4% of the radioactivity was in chromatographic fractions eluted with 1.5% acetone, and 5–10% was in more polar compounds which were eluted with 100% acetone. These latter fractions were not characterized, but probably are composed mainly of oxidized contaminants of the perfusing retinol-C^{14}. The circulation of retinol-C^{14}, β-carotene-C^{14}, or the other substrates through the apparatus for 1 hr in the *absence* of a liver yielded a similar distribution of radioactivity in polar fractions from alumina columns.

Formation and Release of Metabolites of Retinol

Neither retinol-C^{14} nor retinol-C^{14} ester was released from livers during perfusion under the experimental conditions employed. Similarly, labeled retinol was not released during the perfusion of livers which contained retinol-C^{14} ester as a result of previous injections of retinol-C^{14} into the intact rat. The addition to the perfusate of 5 ml of serum from retinol deficient rats did not induce retinol release.

However, water-soluble metabolites of retinol were excreted in the bile from livers during perfusion. In five experiments in which the bile flow during a 1 hr perfusion period was 0.12–0.25 ml, 5.5–10.2% of the original perfusate retinol-C^{14} appeared in the bile. The amount of radioactivity excreted was roughly proportional to the bile flow. Similar amounts of water-soluble C^{14} metabolites also appeared in the bile when retinoic acid-C^{14} or retinal-C^{14} were perfused. In all cases less than 5% of this bile radioactivity was extractable with hexane under either acidic or basic conditions.

DISCUSSION

The isolated liver perfused with diluted blood or buffer has been recently employed by others in studying the uptake, metabolism, and release of lipids and lipoproteins (14–19). Although oxygenated buffer rather than blood was perfused in the present study, the color, general macroscopic appearance, and microscopic structure of the liver remained normal during the 1 hr perfusion period, and the bile flow and clearance of bromosulfalein and carbon particles by these livers were satisfactory. Hence, the behavior of these preparations approximates that of the liver in situ for short perfusion periods.

Retinol, retinal, retinoic acid, and retinol acetate were taken up by perfused livers at essentially the same initial rate. Since the rate decreased rapidly during perfusion, a formal kinetic expression of the rate is not possible. In other similar systems in which lipid is removed from plasma or perfusate, however, the initial rate followed a first-order plot (15, 20, 21). With the assumption that the amount of retinol ester formed during a 1 hr perfusion period reflects the initial rate of retinol uptake, the linear relationship between liver retinol ester and the initial perfusate concentration of retinol is also in keeping with a first-order process.

When an average first-order rate constant is calculated for the initial uptake of these four substrates, a value of 0.12–0.16 min^{-1} is obtained.

In contrast, β-carotene is removed at a 20-fold slower rate under similar conditions of perfusion. Since the rate of retinol ester formation is a linear function of the β-carotene concentration in the perfusate (13) and the rate of uptake closely follows a first-order plot (Fig. 1), a first-order process may be presumed with a $k = 0.0056$ min^{-1} (13). In view of the differences in the rate of uptake of carotene and of the retinol derivatives, and of the lack of inhibition of one substrate on the uptake of the other, different absorption sites may well exist in liver for the removal of these compounds. However, this problem clearly requires further exploration.

Retinol and its ester are normally associated with specific proteins and lipoproteins of plasma (22–26), and dietary protein influences the intestinal absorption, liver storage, and plasma transport of retinol and β-carotene (27–29). Nevertheless the uptake and metabolism by perfused liver of retinol and its derivatives which were solubilized in micelles of Tween 80 occurred readily from buffer solution and were unaffected by the addition of rat serum. Although the chylomicron is the physiological form in which dietary retinol and carotenoids are presumably presented to the liver, these experiments demonstrate that plasma protein is not *essential* for the uptake and normal metabolism of retinol and its derivatives by the liver. The need for protein might more reasonably be associated with release of retinol from the liver, and more specifically, with the biosynthesis of a suitable protein carrier within the liver. Retinol was not released into the perfusate when buffer alone was used, nor when serum from normal or retinol deficient rats was present. The synthesis and release of plasma proteins from perfused liver is greatly enhanced by the addition of a complete amino acid mixture to the perfusate (30), however, and the formation and release of lipoproteins seemingly requires de novo protein synthesis (31). Hence, the lack of retinol release in our system may be attributed to unsuitable conditions for protein synthesis.

The major metabolic product of retinol, of retinol acetate, and of retinal in isolated perfused liver is retinol ester. Clearly, liver has the enzymatic capability of esterifying retinol with long-chain fatty acids of endogenous origin. Surprisingly, previous attempts to demonstrate the in vitro esterification of retinol with liver preparations were unsuccessful (32–34), and hence the liver was presumed to store retinol ester, but not to synthesize it. This uncertainty has now been resolved by the present investigation, that of Worker (11), and recent studies on liver retinol esterase by Futterman.[1]

[1] Futterman, Sidney, personal communication.

The amount of liver retinol-C^{14} ester formed was directly proportional to the concentration of perfusate retinol-C^{14} over the range of 3 to 1000 μg/100 ml of perfusate. The slope of this linear relationship is 0.25–0.30 (Fig. 2), that is, 25–30% of the initial dose appeared as liver retinol ester. With respect to the retinol actually removed by the liver, about 50% was found as liver retinol ester (Table 1). Similarly, Worker (11) reported that 40% of the perfusate retinol appeared as retinol and retinol ester in livers perfused for 2 hr with 100 μg of retinol suspended in Tween and saline. Furthermore, 20% of a dose of retinol injected into an intact rat was found as liver retinol ester 2 hr after administration (35). In the bioassay method of Ames and Harris (36), about 67% of the retinol dose was normally found in liver retinol ester over a range of oral doses of 150 to 3000 μg per animal. Since the periods between dosage and analysis were 5 days in the bioassay procedure and only 1–2 hr in the perfusion experiments, the higher value of liver retinol ester found in the former case is not surprising.

The other major products of retinol, clearly demonstrated for the first time in the perfused liver, are a group of water-soluble metabolites which are excreted in the bile. Although polar metabolites of retinol and retinoic acid have been found in small amounts in the plasma, urine, feces (24, 37–39), intestine (40), stomach (41), and liver[2] (42) of chickens and rats, the rapid metabolism and excretion of retinol derivatives in bile has not previously been suspected. Retinol itself was not found in the bile in the present study, and its reported presence there (43) is based on a nonspecific colorimetric assay. The poor over-all recoveries of retinol after oral dosing, the incomplete storage of retinol as liver retinol ester in bioassay procedures, and the appearance of metabolites of retinol in the intestinal tract are all explicable in terms of the observed biliary secretion of appreciable amounts of water-soluble derivatives. These metabolites are currently being characterized, and the possible significance of their enterohepatic circulation (44) is being probed.

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