Liquid–gel partition chromatography of vitamin A compounds; formation of retinoic acid from retinyl acetate in vivo

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Abstract A clear separation of retinol, retinal, and retinoic acid has been achieved by liquid–gel partition chromatography on Sephadex LH-20 with solvent mixtures of chloroform, Skellysolve B, and methanol. A mixture of retinyl esters, retinol, retinal, and retinoic acid has been resolved on hydroxyalkoxypropyl Sephadex using Skellysolve B and acetone. There is no decomposition of any of the vitamin A compounds during chromatography, and recovery is complete. The combination of mildness and potential for resolution makes liquid–gel partition chromatography a superior tool for the separation of vitamin A compounds. This method has been applied to the study of vitamin A metabolism at physiological levels in the vitamin A-deficient rat. Retinyl palmitate, an ester of retinoic acid, retinal, retinol, retinoic acid, and a polar metabolite have been demonstrated in various tissues of the rat 12 hr after a dose of 2 μg of [11-^{14}C]retinyl acetate.

Supplementary key words retinol · retinal · retinyl esters · Sephadex LH-20 · hydroxyalkoxypropyl Sephadex · rat

The lability of vitamin A compounds has always presented a challenge to the investigator interested in vitamin A. In the past, adsorption on alumina has been the most widely used chromatographic method for the separation and purification of vitamin A compounds. Other adsorbents, such as silicic acid, silica gel, and dicalcium phosphate have also been used, but to a lesser extent, while separation methods such as partition and ion exchange chromatography have been applied to specific problems in the vitamin A field (1). However, incomplete recovery (1–4), incomplete resolution of vitamin A compounds (1, 3, 4), and formation of artifacts (1, 4–6) are some of the drawbacks experienced that have often hampered the investigation of vitamin A metabolism.

Liquid–gel partition chromatography has been used extensively for the study of lipid-soluble substances (7–11). The recent success in the application of Sephadex gel chromatography to the study of vitamin D metabolites (12) has led us to investigate its usefulness for the separation and purification of vitamin A compounds. In this paper we report the separation of synthetic mixtures of vitamin A compounds by liquid–gel partition chromatography.

The physiological occurrence of retinoic acid in animal tissues has often been questioned (13, 14), even though this compound is biologically active (15, 16) and is found in animal tissues and bile after the administration of large amounts of vitamin A compounds (17–19). Recently, the in vivo formation of retinoic acid from microgram quantities of administered retinol or retinyl acetate has been demonstrated (6, 20–22), but the separation methods used have not been satisfactory.

Using the gel methods, the presence of [^{14}C]retinoic acid as well as other ^{14}C-labeled vitamin A compounds in the tissues of rats after a physiological dose of [^{14}C]retinyl acetate can be unequivocally demonstrated.

EXPERIMENTAL METHODS

Animals

Weanling male albino rats weighing 50–55 g (Holtzman Co., Madison, Wis.) were housed individually in hanging wire cages and fed a vitamin A-free diet (23) ad lib. The animals reached a growth plateau after about 6 wk on this diet, and they were used for experiments when their weight began to decline.

Chemicals and apparatus

[11-^{14}C]Retinyl acetate (sp act 89,760 dpm/μg) was a gift from the Philips-Duphar Co., Weesp, the Netherlands, and was purified immediately before use on a HAPS column, using a solvent mixture consisting of Skellysolve B–acetone 92:8, until the ratio of dpm to micro-

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Abbreviations: HAPS, hydroxyalkoxypropyl Sephadex; EDTA, ethylenediaminetetraacetic acid, as sodium salt; BHT, butylated hydroxytoluene.

grams reached a constant value. [11-^{14}C]Retinoic acid (sp act 9680 dpm/μg) was also a gift from the Philips-Duphar Co., and it was purified on a Sephadex LH-20 column, using a solvent mixture of chloroform–Skellysolve B–methanol 65:35:0.1. All-*trans*-retinoic acid, retinol, retinal, and retinyl acetate were purchased from Distillation Products, Inc., Rochester, N.Y. Retinyl palmitate was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Sephadex LH-20, particle size 25–100 μm, was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. HAPS was synthesized by the method of Ellingboe, Nyström, and Sjövall (11).

Skellysolve B represents the petroleum fraction that distills at 67–68°C. All other solvents and chemicals were reagent grade.

Determination of radioactivity

Radioactivity was determined by liquid scintillation counting in a Packard Tri-Carb model 3375 scintillation counter equipped with an external standard system.

Samples from extraction procedures and column chromatography were evaporated to dryness with a stream of air and counted in a 1:1 mixture of methanol and toluene counting solution (2,5-diphenyloxazole, 2 g; 1,4-bis-2-[4-methyl-5-phenyloxazolyl]-benzene, 100 mg; and toluene, 1 l). The efficiency was 58–65%. For studies of total radioactivity, tissues were combusted in a sample oxidizer, Packard model 305. The $^{14}CO_2$ was dissolved in a mixture of 5 ml of ethanolamine, 9 ml of methanol, and 5 ml of scintillator solution (2,5-diphenyloxazole, 15 g; *p*-bis-[*O*-methylstyryl]-benzene, 0.5 g; and toluene, 1 l) and counted in the liquid scintillation counter. The efficiency was 49–57%.

Preparation of animals

Under ether anesthesia, rats were surgically cut and injected intrajugularly with 50 μl of ethanol containing 2 μg of [11-^{14}C]retinyl acetate. The tissues from 12 rats were pooled for each experiment.

Extraction of tissues

Tissues (1–10 g) were homogenized with 2 parts of water, containing 0.5 mg of vitamin C and 0.5 mg of EDTA per ml, using a Waring Blendor (model 5011). The homogenate was lyophilized overnight, and the residue was then extracted twice with 30 ml of chloroform–methanol 1:1, containing 50 μg of BHT/ml. The precipitate was further extracted twice with 20 ml of methanol containing 50 μg of BHT/ml. The extracted precipitate was combusted, and the radioactivity remaining in it was determined as described above. Supernates were combined and filtered through Whatman no. 50 filter paper. The filtered extracts, representing the tissues of several rats, were divided in several parts to be used for column chromatography in different systems. Unlabeled vitamin A carrier compounds were added at this time.

Preparation of samples for chromatography

The extract (100–500 ml) was evaporated in a rotary evaporater, and the final combined extracts were centrifuged. The supernate was dried with a stream of nitrogen, dissolved in 0.5 ml of the solvent, and used for chromatography.

Column chromatography

Sephadex LH-20, 20 g, was slurried with 100 ml of the appropriate solvent (chloroform–Skellysolve B–methanol, either 65:35:0.1 or 65:35:1). HAPS, 20 g, was slurried with 100 ml of Skellysolve B–acetone 92:8. After 48 hr of equilibration in the cold room, the slurry was poured into a 60 × 1.1 cm glass column to a height of 55 cm. At least 50 ml of the solvent was passed through the column before the sample was applied. Chromatography was carried out in the cold room, using a nitrogen pressure of 1–2 psi for the Sephadex LH-20 columns and 0.25–5 psi for the HAPS columns, resulting in a flow rate of about 0.5 ml/min. In order to prevent oxidative degradation of compounds during chromatography, the elution solvents contained BHT, 50 μg/ml. All operations for extraction and

TABLE 1. Radioactivity extracted from tissues[a]

	A After the Addition of Radioactive Compounds at Homogenization		*B* After an Intravenous Dose of 2 μg of [11-^{14}C]Retinyl Acetate to Vitamin A-deficient Rats					
	Liver[b]	Liver[c]	Kidney	Liver	Blood	Skin	Small Intestine	Testes
	%	%	*% of homogenate radioactivity*					
$CHCl_3$–MeOH (1:1) extract	99.8	99.7	90.7	90.1	89.4	91.1	69.8	76.6
Methanol extract	0	0	8.4	8.7	9.9	8.1	27.3	22.7
Total extract	99.8	99.7	99.1	98.8	99.3	99.2	97.1	99.3
Precipitate	0.2	0.3	0.9	1.2	0.7	0.8	2.9	0.7

[a] Results are averages of three experiments in each of which the entire tissue was used except for skin (10-g samples) and blood (5-m samples).

[b] 0.1 μg of [11-^{14}C]retinyl acetate was added to the liver of a stock rat at homogenization.

[c] 0.1 μg of [11-^{14}C]retinoic acid was added to the liver of a stock rat at homogenization.

column chromatography were carried out at cold-room temperatures, under nitrogen atmosphere, and were shielded from light whenever possible.

Identification of compounds

The elution positions of vitamin A compounds were established by chromatographing individual compounds as well as mixtures of authentic compounds in all the chromatographic systems used and examining the ultraviolet and visible absorption spectra of the eluted compounds in a Beckman DGB recording spectrophotometer. The following extinction coefficients ($E^{1\%}_{1\,cm}$ in ethanol) were used: retinoic acid, 1510 at 350 nm; retinol, 1832 at 325 nm; retinal, 1530 at 380 nm; retinyl acetate, 1560 at 325 nm; and retinyl palmitate, 940 at 325 nm.

Hydrolysis of esters

After chromatography, appropriate fractions were evaporated to dryness, dissolved in a mixture of 5 ml of ethanol, 0.5 ml of 50% KOH, and 100 mg of sodium ascorbate (24), and saponified for 30 min by refluxing in a water bath at 70–80°C under nitrogen. The reaction mixture was neutralized with 0.1 N HCl and extracted twice with 10 ml of Skellysolve B. The extract was concentrated and chromotographed.

Calculation of the amount of vitamin A compounds found in tissues

The amount of retinoic acid, retinol, retinyl palmitate, and an ester of retinoic acid was calculated from the radioactivity appearing in the appropriate fractions after column chromatography. The average of values obtained in several experiments was divided by the specific activity of the retinyl acetate and the number of animals used in each experiment, and the results are expressed as nanograms of compound per total tissue from one animal.

RESULTS

Recovery of radioactivity from the extraction procedure

Our modified lipid extraction procedure resulted in excellent recoveries of added retinoic acid and retinyl acetate. Almost all of the radioactivity from retinyl acetate (99.8%) and retinoic acid (99.4%) was found in the chloroform–methanol extracts of the lyophilized liver homogenate (**Table 1**, part A). Of the radioactivity located in tissues after an intravenous administration of labeled retinyl acetate, 70–91% was chloroform–methanol soluble; the remaining radioactivity was more polar and required methanol for extraction. The small intestine and testes contained about 25% of their radioactivity in the more polar fraction. The amount of radioactivity remaining in the residue was less than 1%, with the exception of small intestine (2.9%). Thus, the combination of the two extraction steps resulted in the recovery of 97–99% of the radioactivity from all tissues studied (Table 1, part B).

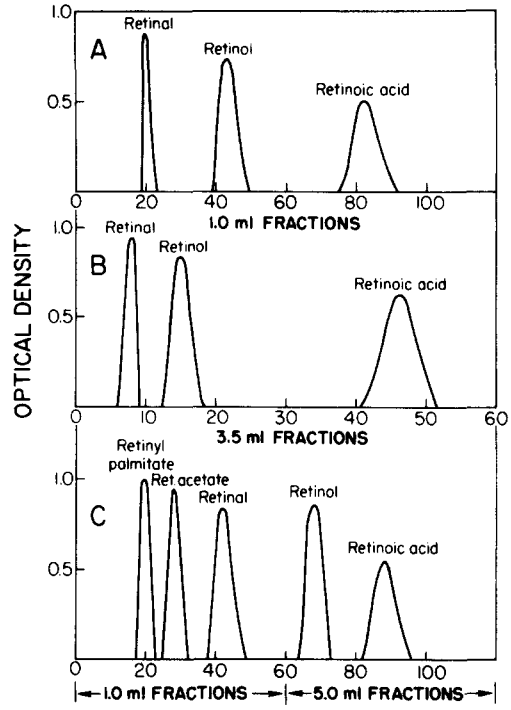

Fig. 1. Chromatography of synthetic mixtures of vitamin A compounds on Sephadex LH-20 and hydroxyalkoxypropyl Sephadex. System *A:* Sephadex LH-20; solvent, chloroform–Skellysolve B–methanol 65:35:1. System *B:* Sephadex LH-20; solvent, chloroform–Skellysolve B–methanol 65:35:0.1. System *C:* hydroxyalkoxypropyl Sephadex; solvent, Skellysolve B–acetone 92:8.

Chromatographic systems for the separation of synthetic mixtures of vitamin A compounds

The most successful systems for the separation of vitamin A compounds on Sephadex LH-20 and on HAPS are illustrated in **Fig. 1.** The use of a solvent mixture containing chloroform–Skellysolve B–methanol in the proportion 65:35:1 (system *A*) resulted in a good separation of a mixture of 15 μg of retinal, 25 μg of retinol, and 40 μg of retinoic acid on the Sephadex LH-20 column, and the same solvents in the proportion 63:35:0.1 provided a good separation of a mixture of 20 μg of retinal, 25 μg of retinol, and 200 μg of retinoic acid. The recovery of all compounds was at least 99%. However, in these systems retinyl acetate and retinyl palmitate elute with retinal.

A solvent mixture consisting of Skellysolve B–acetone 92:8 on a column of HAPS was found to be the best system for routine separation of a mixture of retinyl palmitate, 20 μg; retinyl acetate, 20 μg; retinal, 20 μg; retinol, 70 μg; and retinoic acid, 200 μg (Fig. 1*C*). Since in this system retinol and retinoic acid elute as broad peaks, the fraction volume in the elution area of these substances was

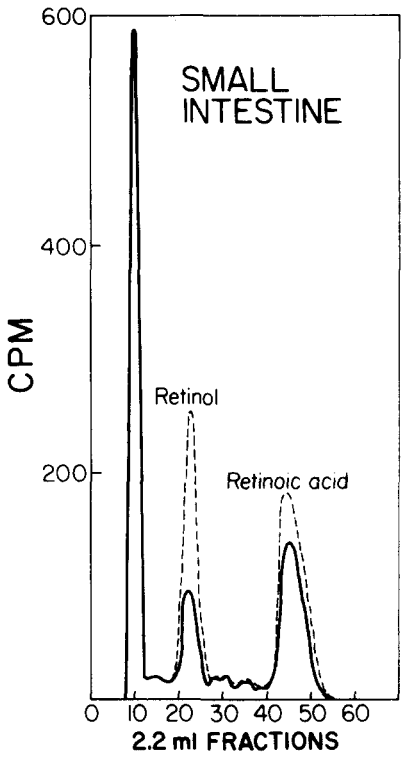

Fig. 2. Chromatography of in vivo formed radioactive vitamin A compounds on Sephadex LH-20. Solid lines represent radioactivity; broken lines, absorbance at the wavelength maximum appropriate for each marker compound. The solvent system is described in Fig. 1*A*. Other details are provided in the text.

increased from 1 ml to 5 ml. The recovery of vitamin A compounds in this system was 99%, except for retinol (98%) and retinoic acid (96%).

Recovery of vitamin A compounds from the entire isolation and separation procedure

When [11-^{14}C]retinoic acid (0.9 μg) and unlabeled retinoic acid (50 μg) were added to liver, kidney, or small intestine at homogenization, carried through the isolation procedure, and chromatographed using the system described in Fig. 1*A*, the recovery was 98.7%. In a similar study with 0.1 μg of [11-^{14}C]retinyl acetate and 25 μg of unlabeled retinyl acetate, the recovery was 99.1% when using the chromatographic system described in Fig. 1*A* and 98.5% when using the system described in Fig. 1*C*.

Separation of in vivo formed radioactive vitamin A compounds on Sephadex LH-20 and hydroxyalkoxypropyl Sephadex columns

The Sephadex LH-20 chromatographic system was applied to tissue extracts obtained from vitamin A-deficient rats that had been injected with 2 μg of [11-^{14}C]retinyl acetate 12 hr prior to being killed. A mixture of unlabeled carrier vitamin A compounds was added to the extracts, as in Fig. 1*A*. The resulting chromatographic profiles for the vitamin A compounds were similar for the various tissues and are exemplified by the small intestinal profile of **Fig. 2.** All tissues studied were found to contain retinyl esters, retinol, and retinoic acid. The amount of retinyl esters in the blood was very low. In most tissues there was proportionally more retinol than retinoic acid, except in the small intestine and liver, where the trend was reversed. The above tissue extracts were also chromatographed on HAPS columns. The unlabeled carrier compounds added were retinoic acid, retinol, retinal, retinyl palmitate, and retinyl acetate, as in Fig. 1*C*. The elution patterns of the various radioactive compounds are illustrated in **Fig. 3.** This chromatographic system resolved into several components the single peak (fraction no. 10, Fig. 2) that was isolated using the Sephadex LH-20 system. This fraction was found to consist of at least four components; the first (peak 1a) eluted with most of the yellow lipid of the tissues, while the second, third, and fourth cochromatographed with retinyl palmitate, retinyl acetate, and retinal, respectively (peaks 1b, 1c, and 1d).

Retinyl palmitate or esters eluting in this fraction were the predominant esters in all tissues, while the amount of retinyl acetate detected was insignificant. All tissues examined also contained a nonpolar component (peak 1a), retinol (peak 2), retinoic acid (peak 3), and a small amount of retinal (peak 1d). In the blood, the predominant forms of known vitamin A compounds were retinol and retinoic acid, but small, identifiable amounts of the other compounds were also present.

The more polar metabolites still remaining on the column were eluted by the application of methanol–acetone 1:1 to the column at fraction no. 115. All tissues and blood were found to contain a large amount of their radioactivity in this polar fraction (Fig. 3, fraction 120).

When the first peak (1a) was isolated from liver and kidney, hydrolyzed, and then rechromatographed, 82% and 78% of the radioactivity, respectively, eluted together with retinoic acid, with very little radioactivity remaining in the original area. This suggests that peak 1a is an ester of retinoic acid, possibly containing cholesterol (25, 26).

The second peak that cochromatographed with retinyl palmitate (peak 1b) was isolated from liver, kidney, small intestine, and testes and similarly subjected to hydrolysis. Upon rechromatography it was found that 84–95% of the radioactivity had moved to the position of retinol, suggesting that it had been retinyl palmitate or some other long-chain fatty acid ester of retinol.

The radioactivity isolated from the various peaks with the two chromatographic systems (Figs. 2 and 3) was expressed in nanograms, and the data are shown in **Table 2.** This comparison demonstrates that the amount of compounds isolated from the tissues was about the same for both chromatographic systems, the resolution with the HAPS system in the elution area of the esters being superior to that of the Sephadex LH-20 system.

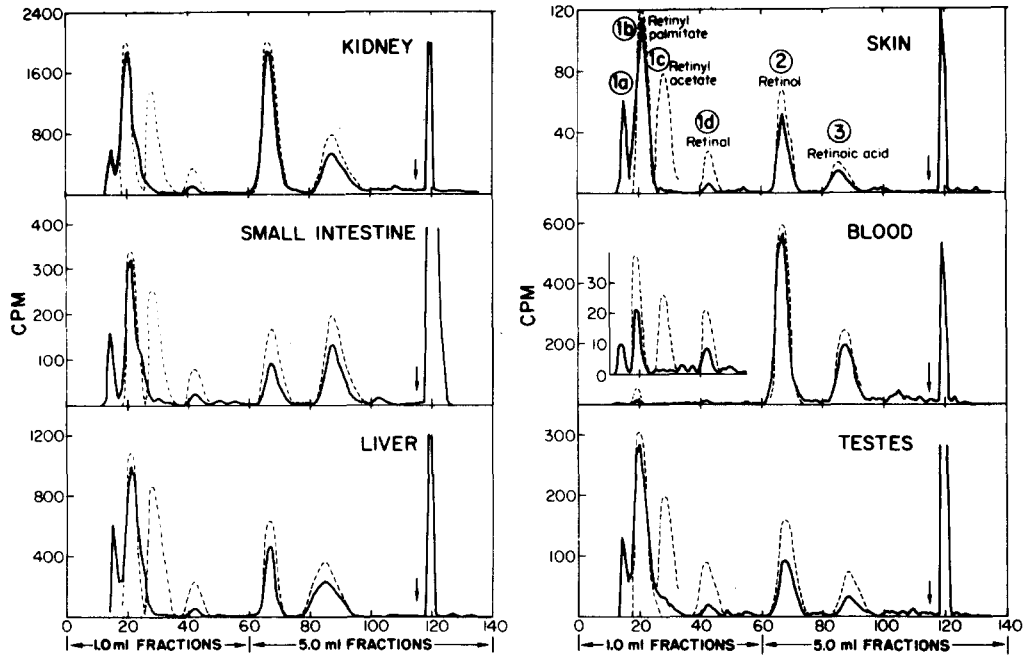

Fig. 3. Chromatography of in vivo formed radioactive vitamin A compounds on hydroxyalkoxypropyl Sephadex. Solid lines represent radioactivity; broken lines, the absorbance at the wavelength maximum appropriate for each marker compound. Solvent system is the same as in Fig. 1*C*. Arrow at fraction no. 115 indicates the position at which the elution was continued with a solvent mixture of methanol–acetone 1:1.

In the skin and liver, the predominant form of vitamin A was in the large ester fraction (peak 1b); in the blood, testes, and kidney, retinol was the major component. A major portion of the radioactivity of each tissue was found to be in the polar fraction, which contained 17.8%, 18.4%, 18.3%, 30.4%, 21.3%, and 65.3% of the radioactivity isolated from skin, blood, kidney, testes, liver, and small intestine, respectively.

DISCUSSION

The main concern of our work here has been the development of reliable isolation procedures for the study of metabolism of vitamin A. In our search for good lipid extraction methods, we experimented with the chloroform–methanol method of Bligh and Dyer (27), which is an adaptation of the method of Folch, Lees, and Sloane Stanley (28). Although the procedure (27) was adequate for the isolation of most of the vitamin A compounds, only 84% of retinoic acid could be extracted into the organic phase. Similarly, after the injection of radioactive precursors and the extraction of tissues by this method, significant amounts of radioactivity remained in the aqueous phase. To ensure complete and safe extraction of all vitamin A compounds, we have modified the above method by the inclusion of both lipid- and water-soluble antioxidants and

TABLE 2. Radioactive vitamin A compounds isolated after administration of radioactive retinyl acetate

Compound	Skin		Blood		Kidneys		Testes		Liver		Small Intestine	
	A[a]	B[b]	A	B	A	B	A	B	A	B	A	B
	ng/animal/total tissue[c]											
(1a) Ester of retinoic acid	[18.1]	3.6	[3.3]	0.7	[64.2]	8.6	[13.3]	2.6	[81.7]	15.2	[11.4]	2.0
(1b) Retinyl esters		14.2		1.7		52.6		10.6		60.7		10.4
(1d) Retinal		0.4		0.9		2.9		0.5		2.2		0.4
(2) Retinol	7.0	6.5	114.3	118.4	80.4	78.5	5.1	4.8	23.0	22.0	3.0	3.0
(3) Retinoic acid	2.8	2.7	35.4	37.4	36.1	32.0	1.6	1.5	32.7	35.0	6.6	6.2
Polar compounds		7.0		42.5		40.1		10.4		42.5		42.6

Numbers in brackets are sums of ester of retinoic acid, retinyl esters, and retinal. Numbers in parentheses represent corresponding peaks in Fig. 3.

[a] A = data from the chromatography of in vivo formed radioactive vitamin A compounds on Sephadex LH-20. See also Fig. 2.

[b] B = data from the chromatography of in vivo formed radioactive vitamin A compounds on HAPS. See also Fig. 3.

[c] See paragraph on calculations.

by the removal of tissue water by lyophilization prior to extraction. Since it has been reported that retinoic acid in a bicarbonate buffer is rapidly degraded in the presence of Ca^{2+}, Mg^{2+}, Ba^{2+}, or Cu^{2+} (29), we also added a metal chelator, EDTA (as disodium salt), to the tissues at homogenization. Furthermore, after the extraction of the tissue with chloroform–methanol, the tissue is finally extracted with methanol, which solubilizes the components that are more polar than retinoic acid and retinoic acid that might be bound to tissues. The extraction method reported here results in excellent recovery of all the compounds studied.

The liquid–gel partition chromatography described in this paper is the first application of this type of column chromatography to the separation of vitamin A compounds and is also the first chromatographic system to date that combines excellent recovery of vitamin A compounds with excellent resolution. Aside from the practical advantages (the columns are easy to prepare, can be reused several times, and require less solvent than conventional adsorption columns), the most important merit of these partition columns is that they do not artifactually alter the vitamin A compounds nor do they bring about degradation.

Recoveries of 15–200-μg amounts of vitamin A compounds approximate 100% in all cases. In addition to the precautions described, careful attention was paid to avoiding light, oxygen, and heat during the isolation procedures.

The best system for routine separation of various vitamin A compounds was found to be that of hydroxyalkoxypropyl Sephadex described in Fig. 1*C*, which gives a clear resolution of retinyl palmitate, retinyl acetate, retinal, retinol, and retinoic acid. The Sephadex LH-20 systems, described in Figs. 1*A*, 1*B*, and 2, do not resolve the esters and retinal, all of which elute together with most of the yellow tissue lipids in the early part of the chromatography. This system, however, suggests potential for the resolution of retinol, retinoic acid, and more polar vitamin A metabolites.

In our studies on the metabolism of retinyl acetate at physiological levels with the hydroxyalkoxypropyl Sephadex system, it was possible to demonstrate the in vivo occurrence in all tissues of an ester of retinoic acid and to separate it from the long-chain fatty acid esters of retinol (Fig. 3). This ester may be the cholesteryl ester of retinoic acid, which has been found in the lymph (26) and in the small intestine (25) after the administration of retinoic acid. Nonacidic lipids derived from retinoic acid have been reported also in other tissues and are thought to represent retinoic acid in ester linkage (30).

The predominant form of vitamin A ester was found to be associated with the fraction containing retinyl palmitate. It is very likely that this fraction also contains retinyl esters of other long-chain fatty acids such as retinyl stearate, which has been shown to be an important component of vitamin A esters in all tissues (31).

All tissues examined contained retinol, retinoic acid, an ester of retinoic acid, and a small but significant amount of retinal. Furthermore, the introduction of a second, more polar solvent after the elution of retinoic acid resulted in the appearance of a polar peak that was common to all tissues studied. These findings confirm the previous studies from our laboratory that demonstrated retinoic acid to be a normal metabolite of retinol in the liver, intestine, and bile (20) and of retinyl acetate in the kidney and blood (6). However, several radioactive components isolated in these studies have remained unidentified.

Our findings do not agree with those of Goodman, Huang, and Shiratori (31), who failed to demonstrate any retinoic acid, retinal, or esters of retinoic acid after a 54-μg dose of [15-^{14}C]retinyl esters (mainly as palmitate) administered intravenously in the form of chylomicrons. In subsequent studies from the same laboratory (26), the administration of a small (7–14 μg) dose of [^{14}C]retinol resulted in the formation of small amounts of polar acidic metabolites, which, although not characterized, were presumed to include also retinoic acid. McLean (21) and Jones (22) have studied the metabolism of small, physiological doses of retinyl acetate. Retinol was found to be the predominant form of vitamin A in most tissues, and esters comprised a lesser fraction of the total. Retinoic acid was identified only in the liver and retinal was found only in the eye, with no new metabolites detected in any tissues. However, significant amounts of unidentified radioactive compounds were detected in various other fractions after alumina chromatography.

It is interesting to note that in the kidney the predominant form (35.8%) of vitamin A is retinol (Table 2), a finding in agreement with the results of Jones (22). Both the liver and the small intestine had more retinoic acid than retinol. A significant amount of retinoic acid was found also in the testis, a tissue that is unable to utilize this form of vitamin A for its reproductive function (32). In the blood, the predominant forms of vitamin A were retinol and retinoic acid.

A major component of all tissues studied and also of blood was eluted in the polar fraction. The radioactivity of this fraction constituted 20–60% of the total radioactivity isolated from the tissues. It is possible that this fraction might contain the unidentified polar metabolites reported in previous studies (2, 22), the carboxylic acid metabolite of vitamin A (33), and the polar metabolites of vitamin A reported to be associated with sugar transport (34–36). Furthermore, this fraction might also include the glucuronide derivative of retinoic acid (37), especially in the small intestine, where this polar metabolite was the predominant radioactive compound.

In the experiments described here, we have used vitamin A-deficient animals in the expectation that the ad-

ministered radioactive vitamin A would be used more efficiently by those depleted tissues that require this vitamin for their function and thus a maximum conversion into metabolites would take place. Studies from our laboratory have shown (38) that a small injected dose of retinyl acetate is metabolized *very slowly* in the vitamin A-deficient rat, probably because of a strict conservation mechanism. This has also been reported by Jones (22). On the other hand, in the presence of large available liver stores of vitamin A, such as would be expected to exist in a normal rat, more metabolites would be produced. However, in that case the administered radioactivity would be diluted by the tissue stores of vitamin A and its metabolite pools, making interpretation of metabolism studies very difficult, and, additionally, might render the amount of particular labeled metabolites too small for detection by present methods.

Recent studies (39) suggest that there is no single, "normal" metabolic pathway for vitamin A and that alternate pathways exist under varying physiological conditions. The rate and pathway of vitamin A metabolism is regulated mainly by the availability of vitamin A reserves in the body: the more vitamin A available, the more metabolized (39, 40), but not necessarily by the same pathway. Thus, newly absorbed vitamin A in the normal rat may enter a pool in which it is preferentially metabolized (39), while in the vitamin A-deficient animal, the vitamin may be metabolized slowly, being functionally bound to receptors.

The intravenous injection of retinyl acetate in an alcoholic solution is not a physiological route of entry of this vitamin in the circulation, but it is a widely accepted method for quantitative time-course studies of dose–metabolite relationships. Vitamin A compounds when administered this way are able to stimulate such functions as RNA synthesis in the intestine (41) and liver (42).

Although the observed metabolite pattern may be somewhat different from that of a normal rat, we believe that the vitamin A-deficient rat, as employed in our experiments, provides a good system for the study of location and amount of vitamin A metabolites at the actual sites of function of this vitamin.

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