Retinoylation of proteins in rat liver, kidney, and lung in vivo

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Abstract Retinoylation (retinoic acylation) is a posttranslational modification of proteins occurring in a variety of cell types in vitro. This study was done to examine whether retinoylation occurs in vivo. We found that in retinol-deficient rats, radiolabeled retinol or retinoic acid was incorporated into the liver, kidney, and lung in a form that was not removed by extraction with $CHCl_3$:CH_3OH. About 98% of the radiolabeled retinoid was acid-soluble after digestion with proteinase K indicating that it was covalently bound to protein. About 50% of the retinoid covalently bound to liver and kidney protein was removed by mild hydrolysis with CH_3OH-KOH. Methyl retinoate, all-*trans*-retinoic acid, and polar metabolites of retinoic acid accounted for essentially all of the retinoids released. We conclude that retinoylation of protein occurs in vivo primarily via the formation of an ester bond.—**Myhre, A. M., N. Takahashi, R. Blomhoff, T. R. Breitman, and K. R. Norum.** Retinoylation of proteins in rat liver, kidney, and lung in vivo. *J. Lipid Res.* 1996. **37:** 1971–1977.

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Retinoic acid (RA) nuclear receptors, acting as transcription regulators, mediate many effects of RA (1). However, these receptors may not mediate some effects of RA (2–7). An alternative mechanism may be retinoylation of proteins (8). Retinoylation occurs in a variety of eukaryotic cells that respond to all-*trans*-retinoic acid (tRA) (9–12). In HL60 cells, the dose–response curves for tRA-induced differentiation and retinoylation are similar (8).

At least 20 proteins are retinoylated in HL60 cells. Retinoylated proteins identified are vimentin and the RI and RII regulatory subunits of cyclic AMP-dependent protein kinase in HL60 cells (13, 14) and the cytokeratins in normal human epidermal keratinocytes (10).

While retinoylation has been demonstrated in vitro, its occurrence in vivo has not been reported. The purpose of the present study was to learn whether retinoylation occurs in vivo. Here we show that retinol (ROH)-deficient rats administered either [^{3}H]ROH or [^{3}H]tRA contain RA covalently bound to protein.

MATERIALS AND METHODS

Chemicals

Radioactive ROH ([11,12-^{3}H], 50 Ci/mmol) and tRA ([11,12-^{3}H], 50 Ci/mmol) were purchased from DuPont-New England Nuclear (Boston, MA). Radioinert tRA, phenylmethylsulfonyl fluoride, aprotinin (5–10 trypsin inhibitory units/mL), and sodium dodecyl sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). Activated Raney nickel catalyst was purchased from Janssen Chimica (Beerse, Belgium). All other reagents were of analytical grade.

Animals and tissue preparation

Three-week-old male Wistar rats (Møllegaard), about 50 g, were maintained on a ROH-deficient diet (EWOS-R397) for 12–14 weeks when the animals weighed about 270 g. ROH deficiency was confirmed by a decreased growth rate and a total ROH concentration <500 pmol/g liver tissue.

ROH-deficient rats were given either 0.5 mCi [^{3}H]ROH (10 nmol in 1 mL ground nut oil) intraduodenally 5 or 24 h before they were killed or 0.5 mCi [^{3}H]tRA (10 nmol dissolved in ethanol) intraperitoneally 5 h before they were killed. In some experiments 33 μmol of radioinert tRA was given orally when [^{3}H]ROH was administered.

Abbreviations: tRA, all-*trans*-retinoic acid; RA, retinoic acid; HPLC, high performance liquid chromatography; ROH, retinol; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis.

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Tissues were disrupted on an Ultra Turrax homogenizer and diluted to 20% (wt/vol) in a phosphate-buffered isotonic NaCl solution (pH 7.4) containing 40 µL aprotinin solution/mL and 1 mM phenylmethylsulfonyl fluoride. Homogenates were sonicated for 10 s at 50% output on a VirSonic 50 (The Virtus Co., Gardiner, NY), extracted according to Bligh and Dyer (15), and the delipidized residue was collected by centrifugation at 10,000 *g* for 10 min. The extraction was repeated, usually five or more times, until there was no radioactivity in the supernatant fraction.

The delipidated residue was dried under N_2 gas and then dissolved in 10% sodium dodecyl sulfate. Radioactivity was measured in a liquid scintillation spectrometer.

Protein concentration was estimated according to Lowry et al. (16) with bovine serum albumin as a standard.

HPLC analysis of endogenous ROH and retinyl esters

The ROH status of the animals was monitored by analysis of total ROH in tissues. The retinyl esters in tissue homogenates were hydrolyzed to ROH with 10% KOH in ethanol and then extracted with hexane (17). ROH was separated on a 5-µm Supelcosil LC 8 (250 × 4.6 mm) column (Supelco Inc., Bellefonte, PA) with CH_3OH–H_2O 95:5 (vol/vol) at a flow rate of 1 mL/min (HPLC System I). ROH was detected by absorbance at 328 nm.

HPLC analysis of non-covalently bound [^{3}H]retinoids

Non-covalently bound [^{3}H]retinoids were removed from acidified tissue homogenates with three ethyl acetate extractions (18). The ethyl acetate fractions were pooled, washed with water, and then evaporated to dryness under N_2 gas. The residues were dissolved in methanol and analyzed for retinoids using a 250 × 4.6 mm Suplex pKb-100 column (Supelco Inc.) with a mobile phase of *n*-butanol–acetonitrile–2% ammonium acetate–methanol–glacial acetic acid 2:69:16:10:3 (vol/vol) at a flow rate of 1 mL/min (HPLC System II). The most polar retinoids eluted at 4 min, ROH eluted at 10 min, and tRA eluted at 18 min. [^{3}H]retinoid radioactivity of column fractions was measured by liquid scintillation spectrometry.

Stability and analysis of covalently bound retinoids

Ester bonds were cleaved from the dried delipidated residue, prepared as described above, by incubation with 0.1 N KOH in CH_3OH (methanolysis) (19) for 2 h at room temperature under N_2 gas. The reaction mixture was centrifuged, and the supernatant fraction was acidified and retinoids were removed with three ethyl acetate extractions. The ethyl acetate fractions were pooled, washed with water, and then evaporated to dryness under N_2 gas. The residue was dissolved in a small volume of methanol and analyzed for [^{3}H]retinoids using a 300 × 3.9 mm Nova-Pac C_{18} column (Waters Associates, Milford, MA). Retinoids were eluted with a mobile phase of methanol–1% ammonium acetate in H_2O–acetonitrile 13:39:48 (vol/vol) from 0 to 35 min followed by a linear gradient to 100% acetonitrile between 35 and 45 min and finally with acetonitrile between 45 and 60 min all at a flow rate of 1 mL/min (HPLC System III). [^{3}H]retinoid radioactivity of column fractions was measured by liquid scintillation spectrometry.

The detection of protein-thioethers was carried out with Raney nickel catalyst according to Farnsworth et al. (20). Proteins were digested with proteinase K as described (19).

2D-PAGE

2D-PAGE was performed according to O'Farrell (21) with minor modifications (19). Gels were fixed, stained with Coomassie blue R-250, and prepared for fluorography with Entensify (DuPont/NEN) according to the manufacturer's instructions.

RESULTS

Retinoids bound to tissue proteins in vivo

In the animal, dietary ROH is a major precursor of RA. Initially, we estimated ROH conversion to RA in our ROH-deficient animals. We gave 0.5 mCi of retinol and the recovered radioactivity values in kidney and liver in two animals after 24 h were about 2.5–5.0% (**Table 1**). This rather low recovery is usually found in

TABLE 1. Non-covalently bound [^{3}H]retinoids in liver and kidney of ROH-deficient rats 24 h after receiving [^{3}H]ROH

Tissue	dpm/g (× 10^6)	Percentage of Total dpm Recovered		
		ROH	tRA	Polar Metabolites
		%		
Liver				
Rat 1	1.9	25	36	34
Rat 2	1.1	28	27	35
Kidney				
Rat 1	8.1	30	7	56
Rat 2	27.2	52	2	36

ROH-deficient rats were given 0.5 mCi [^{3}H]ROH. After 24 h, the animals were killed. Non-covalently bound retinoids were extracted with ethyl acetate from portions of the livers and kidneys and analyzed with HPLC System II. The livers of rats 1 and 2 weighed 6.9 g and 7.4 g, respectively. The kidneys of rats 1 and 2 weighed 1.4 g and 1.7 g, respectively. Between 90 and 95% of the radioactivity applied to the columns was recovered in the ROH, tRA, and polar RA metabolite fractions. The retinyl ester concentrations in these ROH-deficient animals were extremely low.

TABLE 2. Retinoylation in liver, kidney, and lung

Tissue	[^{3}H]ROH		[^{3}H]tRA	[^{3}H]ROH + tRA
	5 h	24 h	5 h	5 h
	dpm per mg protein			
Liver	1400 ± 160	1050 ± 320	8100 ± 1550	850 ± 620
Kidney	2550 ± 380	14200 ± 4629	19550 ± 6710	420 ± 280
Lung	550 ± 190	620 ± 340	1500 ± 860	620 ± 230

ROH-deficient rats were given 0.5 mCi [^{3}H]ROH. Covalently bound retinoids in delipidized tissue from liver, kidney, and lung were measured. Some rats also were fed 33 μmol radioinert tRA at the same time that they received [^{3}H]ROH. Values are expressed as mean ± SD (n = 3 animals) in dpm/mg protein.

vitamin A-deficient animals (22). We found, 24 h after the animals had received [^{3}H]ROH, that a large fraction of liver and kidney total noncovalently bound radioactivity was in RA and more polar metabolites. The values for [^{3}H]ROH also were high and presumably could continue to be a source of [^{3}H]RA. Interestingly, the liver contained much more RA than the kidney, whereas the radioactive metabolites dominated in the kidney.

Components in the delipidized residues from liver, kidney, and lung were labeled by [^{3}H]ROH (**Table 2**). About 93–97% of the radioactivity in the organs examined was removed from the organ homogenates by extensive extraction with lipid solvents according to Bligh and Dyer (15). Thus about 3–7% of the radioactivity remained bound, and this fraction did not change significantly with time or when radioactive RA was used instead of ROH (data not shown). At 5 h the highest level of labeling in delipidized residues was in the kidney. The level of labeling in the kidney increased 5.5-fold between 5 h and 24 h. Labeling of the liver and lung residues were about the same at 5 and 24 h. Unfortunately, the high cost of these experiments prevented us from carrying out detailed time course studies. We found with two animals that, 5 h after receiving [^{3}H]ROH, there was a 2.3- and 3.6-fold greater specific activity in the kidney cortex than in the medulla.

We performed two types of experiments to assess whether RA was an intermediate in the covalent labeling of tissues by [^{3}H]ROH. The co-administration of radioinert tRA decreased the labeling of liver and, especially, of kidney by [^{3}H]ROH and, at 5 h, about 3- to 8-fold more [^{3}H]tRA, administered intraperitoneally, was incorporated into the three tissues compared to [^{3}H]ROH given intraduodenally (Table 2). We did not further explore whether the routes of administration played a role in the latter results. Differences in the labeling of different tissues by [^{3}H]ROH or [^{3}H]tRA may also reflect isotope dilution by endogenous ROH and RA. However, the endogenous retinoid levels were below the detection limits of our methods.

TABLE 3. Radioactivity released by alkaline methanolysis from delipidized liver tissue.

Temperature	Radioactivity Released	
	2 h	4 h
°C	*percentage of total*	
20	41 ± 8	45 ± 7
56	58 ± 4	59 ± 6

An ROH-deficient rat was given 0.5 mCi [^{3}H]ROH for 5 h. Portions of the delipidized liver, containing 1340 dpm/mg protein, were suspended in CH_3OH–0.1 N KOH and incubated for 2 and 4 h at 20 and 56°C. Total recovery of radioactivity (released + bound) was between 84% and 104%. Values are expressed as mean ± SD (n = 3 samples).

Site of retinoylation and stability of the covalent bond

Evidence that the retinoid label is covalently bound to protein was obtained from experiments in which the protein in the delipidized tissue was digested with proteinase K. About 98% of the label was converted to an acid-soluble form. Treatment of the residue with CH_3OH:0.1 N KOH released about 50% of the radioactivity (**Table 3**). When we treated delipidized liver tissue with Raney nickel catalyst, only about 10% of the initial radioactivity was released, either before or after alkaline methanolysis.

Identity of the [^{3}H]retinoids bound to protein

Retinoids released by alkaline methanolysis from kidney and liver proteins of a rat given [^{3}H]ROH yielded three major peaks of radioactivity upon HPLC (**Fig. 1A**). The peak eluting very early from the column contained about 36% of the total radioactivity removed from kidney protein and about 6.5% of the total radioactivity removed from liver protein. This peak would contain polar oxidized RA metabolites (e.g., 4-hydroxy-RA and 4-oxo-RA) and was not analyzed further. The radioactive peak eluting at about 30 min comigrated with tRA and contained about 4% of the total radioactivity removed from the kidney protein and about 17% of the total radioactivity removed from the liver protein. The last peak eluting from the column comigrated with methyl retinoate. This peak had about 49% of the total radioactivity removed from the kidney protein and about 63% of the total radioactivity removed from the liver protein.

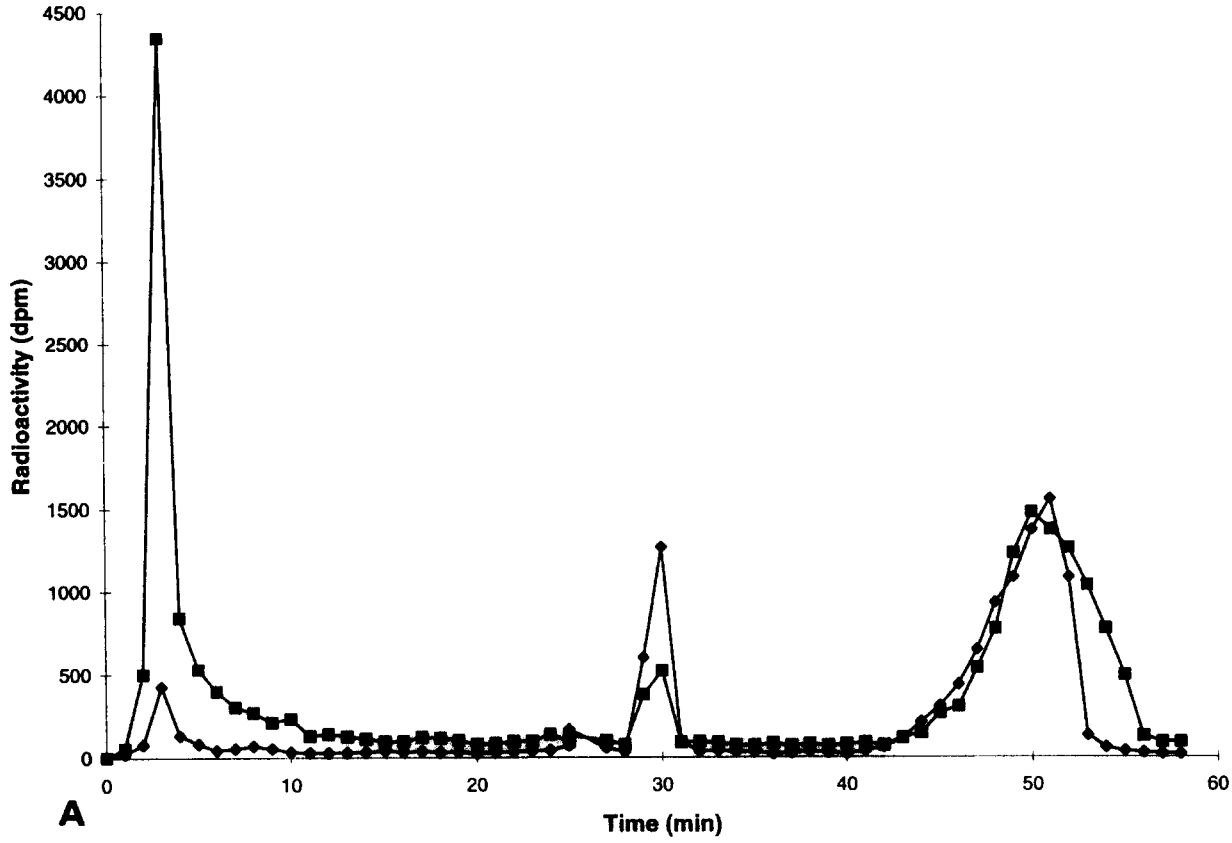

Fig. 1. HPLC separation of radioactive retinoids released by alkaline methanolysis of delipidized liver (◆) and kidney (■) from ROH-deficient rats given either 0.5 mCi [^{3}H]ROH for 24 h (A) or 0.5 mCi [^{3}H]tRA for 5 h (B). Delipidized residues corresponding to 0.1 g of liver and kidney were analyzed. Retinoids were separated with HPLC System III. Fractions of 1 mL were collected. About 85% of the radioactivity applied to the columns was recovered in the polar metabolites, tRA, and methyl retinoate fractions. The methanol used in the experiment shown in Fig. 1A was from a previously opened bottle and was not entirely anhydrous. Thus, some retinoic acid was a product of the hydrolysis. The methanol used in the experiment in Fig. 1 B was from an unopened bottle. Essentially no free retinoic acid was released. Polar metabolites elute from the column after about 4 min, retinoic acid after about 28–29 min, and methylretinoate after 48–50 min.

The radioactivity in these three peaks contained about 87% of the total radioactivity eluted from the columns.

HPLC separation of compounds released by alkaline methanolysis of liver proteins of a rat given [^{3}H]tRA yielded two prominent radioactive peaks (Fig. 1B). The peak eluting early comigrated with oxidized metabolites of RA and contained 40% of the radioactivity applied to the column. The other peak comigrated with methyl retinoate and had 49% of the radioactivity applied to the column.

Labeled proteins visualized by 2D-PAGE

Proteins in the delipidated residues from kidney and liver of a rat given [^{3}H]tRA were separated on 2D-PAGE and analyzed by fluorography. Because of the very low specific radioactivity of the proteins, an extended exposure (18 months) to the film was needed. This resulted in a dark background that made it difficult to see labeled proteins. The most prominent protein, from both liver and kidney, had an M_r value of about 16,000 and a pI of about 6 (**Fig. 2**).

DISCUSSION

One mechanism for the activity of RA in a variety of cell types involves the RA nuclear receptors (RARs and RXRs) (1, 23–27). These receptors are members of multigene families (1) and have specific high affinity binding sites for RA. It generally is accepted that these receptors mediate many actions of RA by activating transcription of target genes after binding to specific DNA sequences.

The role of vitamin A in cell differentiation may not depend exclusively on the nuclear RA receptors. The RA nuclear receptors mediate tRA-induced differentiation of HL60 (28) and P19 embryonal cells (29). However, they are not involved in all aspects of tRA-induced

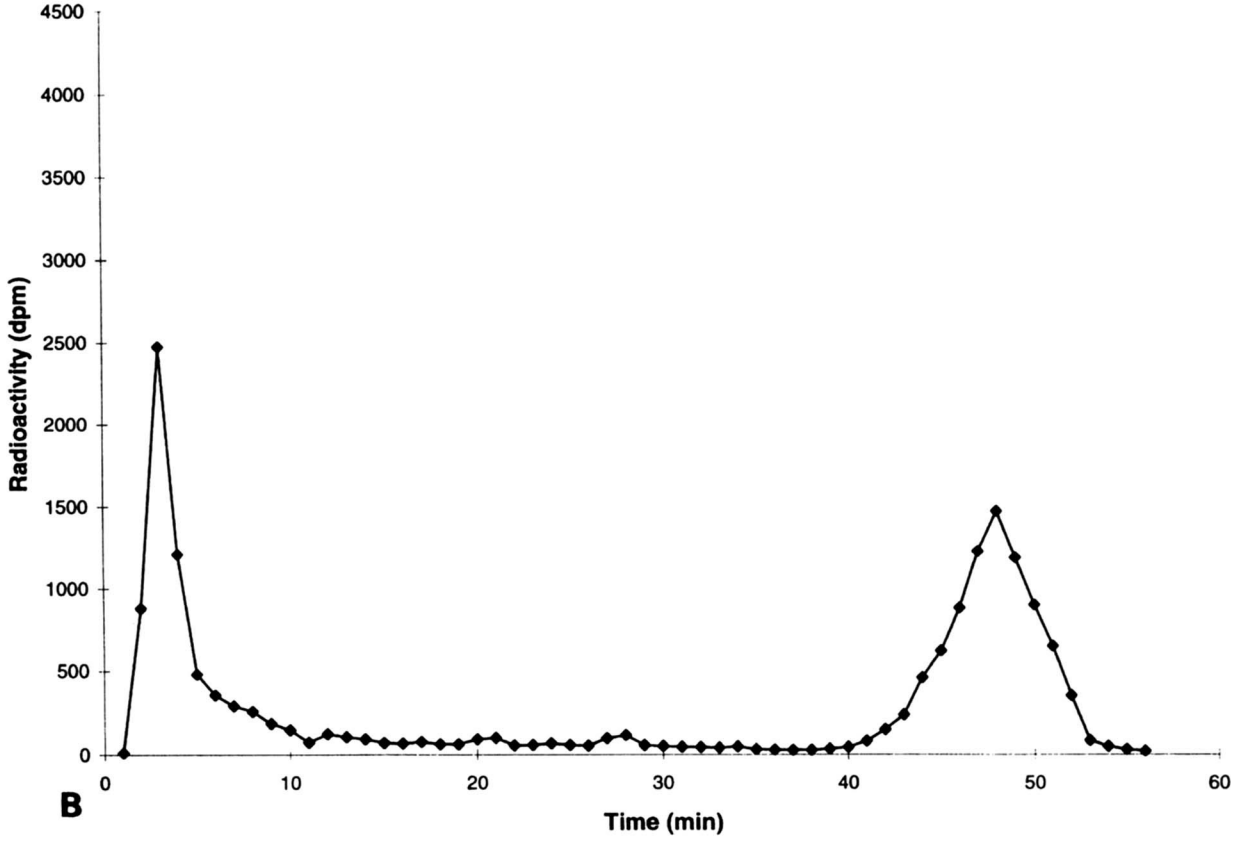

Fig. 1.

differentiation of F9 embryonal carcinoma cells (6, 7) and may function by mechanisms that do not involve their direct interaction with DNA (30, 31). Some effects of tRA are nongenomic (3, 4, 32–34). Therefore, it is possible that other mechanisms, in addition to RA binding to a RA receptor, mediate some RA-induced effects.

It is increasingly recognized that covalent modification of proteins regulates the activity of enzymes, signaling proteins, oncogenes, and transcription factors. Acylation of proteins by RA is a mechanism by which retinoids also may act on cells. RA is covalently bound to proteins of many cell types. The proteins labeled by RA are cell-type dependent (9–12). Furthermore, the nature of the bond between the protein and the retinoyl moiety may vary. Alkaline hydrolysis or methanolysis releases most of the bound retinoid from HL60 cell protein (19). In contrast, retinoylated keratinocyte protein is resistant to these treatments (10). The cAMP-binding regulatory subunits of Type I and Type II cAMP-dependent protein kinases (13), vimentin (14), cytokeratins (10), and ribonucleotide reductase (32) comprise the short list of identified retinoylated proteins.

Retinoylation was shown previously only in vitro. In the current report we demonstrate its occurrence in vivo. [^{3}H]ROH and [^{3}H]tRA were incorporated into rat liver, kidney, and lung in a form that was not removed by extraction with organic solvents (Table

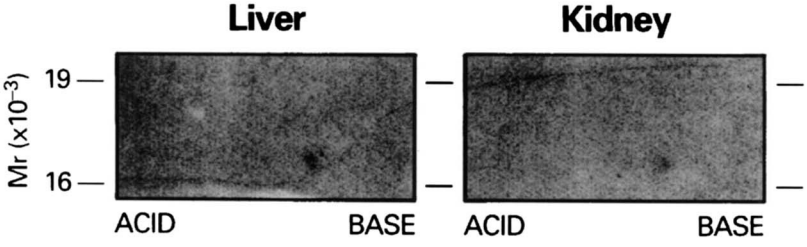

Fig. 2. 2D-PAGE patterns of protein from rat liver and kidney labeled in vivo by [^{3}H]tRA. [^{3}H]tRA (0.5 mCi) was administered intraperitoneally to a ROH-deficient rat 5 h before killing. Delipidized liver and kidney protein were dissolved in isofocusing buffer and separated by 2D-PAGE. We analyzed 1.2 mg of protein from each organ. The specific activities were 8,950 dpm/mg liver protein and 12,665 dpm/mg kidney protein. The gels were prepared for fluorography and exposed to the film for 18 months.

2). Essentially all of the covalently bound [^{3}H]retinoid was acid-soluble after proteolysis demonstrating linkage to protein. Of this radioactivity, >50% was released by alkaline methanolysis (Table 3); >50% of this radioactivity was isolated as methyl retinoate and tRA (Fig. 1). These results are consistent with retinolyation of protein in vivo with the formation of O-ester or thioester bonds.

We do not know the chemical bond or the identity of the retinoid accounting for a substantial portion of the total bound retinoid. Raney nickel catalyst, which cleaves thioethers, released <10% of the total bound retinoid. Thus, a relatively small percentage of the bound retinoid may be in thioether linkage. This linkage is seen for protein isoprenylation (20) and is proposed to explain the stereospecific inhibition of thioredoxin reductase by 13-*cis*-RA (32). Other configurations have been proposed (35) including the binding of RA by an amide bond, analogous to the binding of myristic acid to an N-terminal glycine (36).

It is likely that most, if not all, of the labeling of protein by [^{3}H]ROH is via the formation of RA. Our data supporting this possibility are that ROH is converted to tRA and polar RA metabolites (Table 1), [^{3}H]tRA is more efficiently incorporated into protein than [^{3}H]ROH (Table 2), radioinert tRA decreases the labeling of protein by [^{3}H]ROH, especially in the kidney (Table 2), and methyl retinoate and tRA are recovered from liver and kidney proteins of animals fed either [^{3}H]ROH or [^{3}H]tRA (Fig. 1).

We saw retinoylation in both kidney and liver of a slightly acidic protein with an M_r value of about 16,000 (Fig. 2). Our failure to detect other retinoylated proteins on 2D-PAGE probably is because of the low specific activity of the total labeled protein and the likelihood that radioactivity is distributed among many proteins. Thus, the radioactivity associated with the most highly labeled M_r 16,000 protein may be a relatively small percentage of the total retinoylated protein. Separation of the retinoylated proteins from liver and kidney by techniques that are not as discriminating as 2D-PAGE, e.g., gel or ion-exchange chromatography, may clarify this point.

In conclusion, our results show that retinoylation occurs in vivo and therefore is a widespread phenomenon. Identification of the proteins retinoylated in vivo is a major goal that should aid in gaining a clearer understanding of the role that this protein modification may play in the many actions of RA in biological systems.

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