Retinoyl Beta-Glucuronic Acid: A Major Metabolite of Vitamin A in Rat Bile

Abtsract. *The major metabolite in rat bile of injected C^{14}-retinoic acid was purified by ion-exchange and silicic acid chromatography; it has the spectrum of methyl retinoate, releases retinoic acid upon basic hydrolysis or by treatment with β-glucuronidase, and contains glucuronic acid. The metabolite was characterized by treatment with diazomethane followed by hexamethyl-disilazane, or with periodate followed by semicarbazide, and the products were chromatographed. The metabolite has been tentatively identified as retinoyl β-glucuronide.*

The intermediary metabolism of retinol (vitamin A) in the mammal is not well defined. Ingested retinol may be isomerized (*1*), esterified largely with palmitic acid and stored in the liver (*2*), or oxidized by aldehyde dehydrogenase, aldehyde oxidase, or xanthine oxidase to retinoic acid (*3*). After the injection of radioactive retinal into rats, small amounts of labeled retinoic acid are found in several tissues, but it is found particularly in the bile (*4*). When radioactive retinoic acid is injected into or fed to several species, however, the free acid disappears rapidly from the blood and tissues and is present in trace amounts only after 24 hours (*5*). The major portion of injected retinol, retinal, or retinoic acid is excreted in the bile of bile-cannulated rats within 48 hours (*6*), largely in the form of a more polar acidic product of retinoic acid (*4, 6*). We report here the purification of this major metabolic product of retinoic acid and its tentative identification as retinoyl β-glucuronic acid.

In order to collect suitable amounts of the metabolite for characterization, six white rats, each weighing about 200 g (*7*), were provided with bile cannulas by operation and were injected intraportally with 2 mg of purified 6,7-C^{14}-retinoic acid (400,000 count/min) in 1 ml of 1 percent Tween 40 in Krebs-Ringer bicarbonate buffer, *p*H 7.4. The bile was collected for 24 hours, diluted with four volumes of methanol, and placed on a Bio-Rad AG2-X8 anion exchange resin column. Fractions were eluted with increasing concentrations of acetic acid in methanol (*4*). Fraction III, which contained the bulk of the radioactive material and showed the spectrum of retinoic acid, was further purified on a column of silicic acid which had been prepared by the method of Hirsch and Ahrens (*8*). The column was developed with hexane containing increasing proportions of ethanol, and the eluted fractions were monitored for radioactivity and for optical density at 350 mμ. The major component from the silicic acid column, when chromatographed on a thin-layer plate of silica gel G with a mixture of benzene, chloroform, methanol, and acetic acid (5 : 5 : 5 : 1) as the developing solvent (system I), yielded a single yellow spot with an R_F of 0.45 (*9*). There were only traces of other components as revealed by treatment with iodine, which yields a brown spot with unsaturated compounds. The purified metabolite showed the exact absorption spectrum of methyl retinoate, with a single maximum and little absorption below 300 mμ. Free retinoic acid was rapidly liberated when the metabolite was treated with hot dilute base. The highly purified metabolite clearly contains retinoic acid as a major component.

Since only a small amount of the metabolite was available, its structural aspects were assessed by studying the effects of chemical reagents on its migration rate in thin-layer chromatographic systems. When the solvent used for development was benzene-chloroform-methanol (4 : 1 : 1) (system II) (*10*), the R_F of the metabolite was 0.03. After esterification of the metabolite with diazomethane, the migration rate in this solvent system increased ($R_F = 0.29$), and subsequent treatment of the esterified metabolite with a pyridine solution of hexamethyl-disilazane, which would form silyl ethers of any free hydroxyl groups, further increased the R_F to 0.78. A picture of the iodine-stained thin-layer plate and the radioactivity in various fractions is given in Fig. 1.

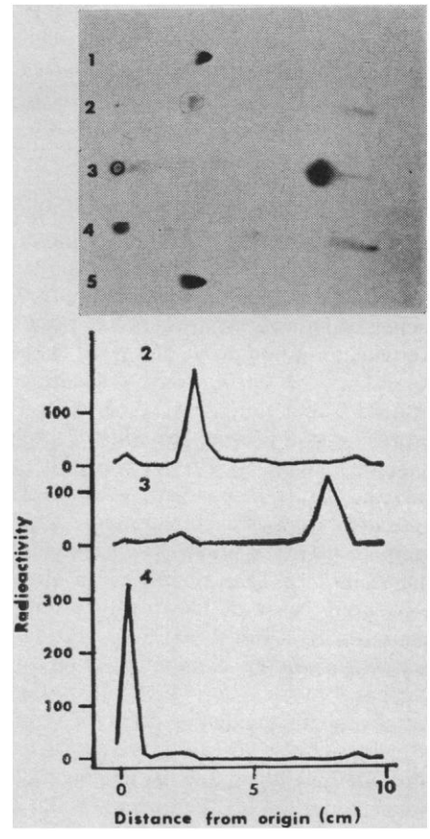

Fig. 1. Chromatographic behavior and radioactivity (count/min) in derivatives of purified fraction III in benzene-chloroform-methanol (4 : 1 : 1). Sample 4 is the untreated metabolite; sample 2, the methyl ester of the metabolite; and sample 3, the silyl ether of the esterified metabolite. Samples 1 and 5 are standards of free retinoic acid.

Upon treatment of the metabolite with sodium periodate, which cleaves *vic*-glycols to dialdehydes, in phosphate buffer at *p*H 6.0, the R_F of the major radioactive peak increased from 0.45 to 0.65 in solvent system I. After incubation of the periodate-treated metabolite with an excess of semicarbazide, which forms semicarbazones with aldehydes or ketones, the R_F was reduced to 0.46. These results suggest that the metabolite contains, in addition to retinoic acid, a free carboxyl group and two or more contiguous hydroxyl groups.

Since lipid-soluble compounds often appear in the urine and bile as β-glucuronides, the metabolite was incubated

Table 1. The effect of saccharolactone on the hydrolysis of the metabolite of retinoic acid and of phenolphthalein β-glucuronide by beef liver β-glucuronidase. The release of retinoic acid and phenolphthalein is expressed as a percentage of the rate in the absence of inhibitor.

Saccharolactone concn.	Rate of hydrolysis (%)	
	Metabolite	Phenolphthalein β-glucuronide
0	100	100
$1 \times 10^{-2}M$	40	39
$4.8 \times 10^{-2}M$		13
$9.6 \times 10^{-2}M$	8	7

in 0.0125*M* acetate buffer at *p*H 4.5 with purified β-glucuronidase of beef liver (*11*), which cleaves acyl as well as acetal bonds. Free retinoic acid was rapidly released by the active enzyme but not by the boiled control. When saccharolactone, presumably a specific inhibitor of β-glucuronidase (*12*), was added, the rate of release of retinoic acid from the metabolite and of phenolphthalein from pure phenolphthalein β-glucuronide was comparable at several concentrations of inhibitor (Table 1).

Finally, the molar ratio of glucuronic acid to retinoic acid in the metabolite, as measured by the naphthoresorcinol test (*13*), was 1.4. The ratio may be greater than unity as a result of small amounts of bilirubin glucuronide which contaminated this preparation. Therefore, we suggest that the major metabolic product of retinoic acid in rat bile is retinoyl β-glucuronic acid (Fig. 2).

The appearance of retinoyl β-glucuronide in the bile is not surprising. Bilirubin and perhaps vitamin K are also excreted as glucuronides in the bile (*14*) and many steroid glucosiduronates are found in urine. Although some glucuronides and glucosiduronates are not absorbed from the gut (*15*), retinoyl β-glucuronide, like estriol β-glucosiduronate (*16*), apparently is absorbed and participates in an enterohepatic circulation (*6*). In normal animals, fraction III is excreted mainly in the feces but also appears in the urine (*17*).

The rapid conjugation of retinoic acid with glucuronic acid in the liver, and perhaps in other organs, excretion of the metabolite in the bile, and its partial reabsorption from the intestine clarify some puzzling aspects of retinoic acid metabolism: the rapid disappearance of free retinoic acid from the liver and other tissues (*5*), and the presence of water-soluble metabolites of retinoic acid in the liver and intestine (*5, 10, 18*). Whether retinoyl β-glucuronide is biologically active per se in growth and metabolism, is cleaved by tissue β-glucuronidases to free retinoate, or is merely an excretion product is still uncertain.

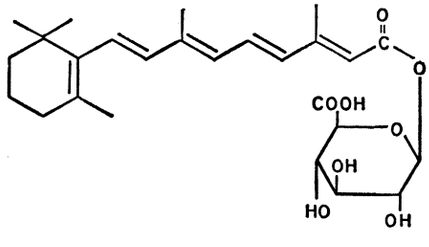

Fig 2. Retinoyl β-glucuronic acid.

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19. We thank O. Wiss of Hoffman-LaRoche Co., Basel, Switzerland, for the 6,7-C^{14}-retinoic acid, G. Wolf of the Massachusetts Institute of Technology for the details of chromatographic system II prior to publication, and D. Tapley of Columbia University for the saccharolactone. R. D. Zachman and Marta Singer assisted during several phases of this investigation. Supported by NIH grant-in-aid AM-1278.

10 December 1964

Vaccinia Virus Directed RNA: Its Fate in the Presence of Actinomycin

Abstract. *RNA similar in base composition to viral DNA is formed in the cytoplasm of HeLa cells infected with vaccinia virus. This RNA is found both in polyribosomes and in a broad 30S to 74S region in a sucrose density gradient. When infected cells are exposed to actinomycin, the amount of this DNA-like RNA in the 30S to 74S region is unchanged. Under the same conditions, 70 percent of the polyribosome-associated DNA-like RNA is degraded to acid-soluble fragments within 2 hours, and protein synthesis is reduced by 90 percent.*

RNA with a base composition similar to that of viral DNA is formed in the cytoplasm of HeLa cells after infection with vaccinia virus (*1*). This RNA, which we call D-RNA, associates with ribosomes to form polyribosomes (*2*), which are the sites of synthesis of viral proteins (*3*). On the basis of these findings and in analogy with other systems (*4*), notably bacteria infected with T-phage (*5*), it seems likely that the D-RNA is the intermediate or "messenger" for viral protein synthesis. In experiments designed to measure the functional half-life of D-RNA, it was found that when RNA synthesis was blocked by the addition of actinomycin D (C_1, IV) to cell cultures, the formation of immunologically detectable viral proteins in infected cells was promptly interrupted. Under similar conditions in uninfected cells protein synthesis continued for several hours (*6*). In an effort to understand the basis for this difference, we have compared the stability of polyribosomes in uninfected and infected cells and found that the bulk of the polyribosomes in infected cells are rapidly degraded when RNA formation is blocked. These studies reveal that a significant fraction of the D-RNA is not in polyribosomes and is not degraded in the presence of actinomycin.

Suspension cultures of 3 to 4 × 10^5 HeLa S3-1 cells per milliliter of growth medium (*1*) were exposed for 1 or 2 hours to uridine-2-C^{14} or P^{32}-phosphate