Retinyl Palmitate, Retinyl Phosphate, and Dolichyl Phosphate of Postnuclear Membrane Fraction from Hepatoma, Host Liver, and Regenerating Liver: Marginal Vitamin A Status of Hepatoma Tissue

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ABSTRACT

The retinyl palmitate content of the postnuclear membrane fraction from 10 Morris hepatomas, their host rat livers, one acetylaminofluorene-induced rat liver hepatoma, and the host liver and of regenerating rat liver was measured by reverse-phase high-pressure liquid chromatography of the chloroform:methanol extracts. Membranes from the hepatoma tissue contained less than detectable levels of retinyl acyl esters, whereas membranes from host liver tissue and regenerating liver contained levels of retinyl palmitate within normal ranges. The amount of cellular retinol-binding protein was also decreased considerably in cytosols from 9618 and 7777 hepatomas. The ratio of endogenous retinyl phosphate to the polysoprenoid dolichyl phosphate available for mannosylation in an assay containing postnuclear membranes and guanosine diphospho[14C]mannose was decreased by a factor of 3 to 10 in hepatoma tissue. Such change in ratio was not attributable to specific changes in retinyl phosphate mannose-synthesizing activity, but it appeared to be related to the vitamin A deficiency condition of the membrane from tumors. As for membranes from vitamin A-deficient liver tissue, postnuclear membranes from rat cystic hepatocarcinoma, Morris 7777, 3924A1-1, and 5123D-1-2 transplantable rat hepatomas and guinea pig line 10 hepatoma all synthesized a mannolipid with intermediate hydrophobic properties between retinyl phosphate mannose and dolichyl phosphate mannose and not normally found in liver tissue. These alterations in patterns of lipid intermediates may be responsible for altered glycosylation of glycoproteins in neoplastic cells. In conclusion, the present investigation establishes that hepatoma tissue are in a status of vitamin A depletion, relative to the host liver.

INTRODUCTION

Vitamin A and its derivatives, collectively termed retinoids, have been implicated in the process of carcinogenesis as protective substances (6, 24).

Recent work has indicated that hepatoma tissue may be depleted of vitamin A. Muto et al. (16) found that most primary human tumor tissue contained less than 1% of the amount of the vitamin compared to the surrounding hepatic tissue, and Büchsel and Reutter (8) could suggest an inverse relationship between 195 and 850 nm. Fluorescence emission at 470 nm was monitored with a Model FS 970 fluorimeter from Schoeffel Instrument Corp., Westwood, N. J., connected in series with the HPLC system, essentially as described previously (4). The UV spectrophotometer was a Hitachi Model 100-30 equipped with a variable wavelength monitor with a Model FS 970 fluorimeter from Schoeffel Instrument Corp., Westwood, N. J., connected in series with the HPLC system, after the spectrophotometer. HPLC columns [Partisil-10-ODS; 4.6 mm (internal diameter) x 25 cm] were purchased from Whatman, Inc., Clifton, N. J. Briefly, the elution program described in Ref. 4 was modified to allow elution of standard retinyl palmitate in 31 min. Solvents were HPLC grade acetonitrile and twice-distilled water. The Partisil column equilibrated with 45% water:55% acetonitrile was eluted with the same starting solvent for 20 min at a flow rate of 1.2 ml/min. At 20 min, the solvent was changed to 98% acetonitrile:2% water until 35 min. At this time, the column was reequilibrated with 45% water until 42 min. A

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2 The abbreviations used are: Ret-P-Man, retinyl phosphate mannose; Dol-P-Man, dolichyl phosphate mannose, Ret-P, retinyl phosphate; Dol-P, dolichyl phosphate; TLC, thin-layer chromatography; DMN-OAC, acetoxydimethyl nitrosamine; AAF, acetylaminofluorene; CRBP, cellular retinol-binding protein.
standard curve was constructed for retinyl palmitate utilizing triplicate injections of 20 ng (10 μl), 50 ng (25 μl), and 100 ng (50 μl) of the retinoid dissolved in ethanol and gave the following respective peak heights (cm) in a typical experiment: 4.2 ± 0.3 (S.D.), 9.8 ± 0.1, and 19 ± 0.1. Alternatively, a second HPLC procedure was used with isocratic elution by 98% acetonitrile and 2% water. Columns of Partisil-10-ODS were standardized each day before analysis of the biological samples with external standard retinyl palmitate in ethanol. In a typical experiment, triplicate injections of retinyl palmitate at 20 ng (10 μl), 50 ng (25 μl), and 100 ng (50 μl) were used, giving respective peak heights of 3.7 ± 0.1, 8.9 ± 1.5, and 17.3 ± 0.01 cm. In this system, the elution time for retinyl palmitate was 21 min at a flow rate of 1 ml/min, and the spectrophotometer range set at 0.05. At the end of each day, the column was washed with acetonitrile (55%):water (45%) and then reequilibrated with the eluting solvent. After 2 to 3 weeks of use, the properties of the stationary phase changed as judged by a reduction in elution time of retinyl palmitate.

TLC was performed on 20-×20-cm precoated silica gel plates (Silica gel 60 F 254) from Merck AG, Darmstadt, West Germany) obtained from Brinkman, Westbury, N. Y. Fluorography was performed on Kodak film XR-5 after it had been sprayed with Enhance (New England Nuclear, Boston, Mass.) following a procedure developed by Bonner and Laskey (7).

Animals and Tumors. Hepatoma-bearing male Buffalo rats were obtained from Drs. Harold P. Morris and Wayne Criss and maintained on a laboratory chow diet until the tumor reached the approximate size of 2 × 2 × 2 cm or equivalent volume. The Morris hepatomas utilized were characterized by Sell and Morris (20) as varying from highly differentiated (9618A, 16, 7787 and 20-1-1), to poorly differentiated (5123tc, 7777) and undifferentiated (9618A, 16, 7787 and 20-1-1), to intermediate (44, 8999, 7800, and 4444) types were a gift of Dr. S. S. Thorgeirsson, National Cancer Institute, according to the protocols of Drs. J. Rice (National Cancer Institute), D. Perantoni and R. Evarts (National Cancer Institute) 24 and 48 hr after partial hepatectomy. The DMN-OAc-transplanted rat hepatoma had been originally induced with intraperitoneal administration of DMN-OAc in male CDF (F-344) rats and used after harvest from a third i.m. transplant, according to the protocols of Drs. J. Rice (National Cancer Institute), D. Kaufman (University of North Carolina), and F. Spangler (Microbiological Associates). Primary tumors of the cystic and solid hepatocarcinoma types were a gift of Dr. S. S. Thorgerisson, National Cancer Institute, and had been induced by the method of Solt and Farber (23) in male Sprague-Dawley rats, using AAF as carcinogen.

Preparation of Postnuclear Membrane Fraction. Animals were starved overnight and killed by bleeding from the neck under ether anesthesia. Livers were homogenized in 2 volumes of ice-cold 0.9% NaCl solution in a glass:Teflon homogenizer. After centrifugation of the homogenate at 2500 × g for 20 min, the resulting supernatant was centrifuged at 105,000 × g for 90 min. The pellet was suspended in a small volume of 0.9% NaCl solution and stored in liquid nitrogen until used. All assays were conducted within 30 days from obtaining the membrane pellets, except as stated.

Transplantable Morris hepatomas were obtained from the s.c. site when they had reached an approximate weight of 6 to 7 g. Tumor tissue utilized was free of gross necrosis and was homogenized as described above for the host liver tissue within 30 to 40 min from the time of resection. Histological sections were prepared to monitor the degree of differentiation, which was consistent with the description of Sell and Morris (20). Monitoring was kindly done by Dr. Karen Hoover, National Cancer Institute.

Regenerating rat liver, guinea pig liver, and other tumor tissue was processed in a similar fashion to obtain the postnuclear membrane fraction.

The postnuclear membrane fraction was utilized to measure retinyl palmitate contents by reverse-phase HPLC. The membrane fraction was suspended in about twice the volume of 0.9% NaCl solution (final volume, 0.2 ml) and extracted with 5 times the total volume (1 ml) of chloroform:methanol (2:1). It was then centrifuged at low speed, and the lower organic phase was removed, dried under nitrogen, redissolved in methanol, and used immediately for HPLC. The limit of sensitivity of the assay was 5 to 10 ng of retinyl palmitate.

The supernatants of the 105,000 × g centrifugation from 7777 and 9618A hepatomas were used for the assay of CRBP as described in the legend to Fig. 2B.

Enzymatic Synthesis of Ret-P-Man and Dol-P-Man from Endogenous Ret-P and Dol-P. The assay system utilized was described recently (11). The incubation mixture contained 0.2 μCi of GDP-[14C]mannose (5 μM) or, as stated, 4 μg bovine serum albumin per ml, 30 mM Tris-HCl buffer (pH 8), 5 mM MnCl₂ or, as stated, 8 mM NaF, 2 mM ATP, 5 mM AMP, and about 1 mg of the microsomal protein in a final volume of 200 μl. Under these conditions, the Km for GDP-mannose was 18 μM for Ret-P-Man synthesis and 1.7 μM for Dol-P-Man synthesis (21). After incubation, lipids were extracted by either of 2 procedures. In Procedure 1, to the mixture were added 5 volumes (1 ml) of chloroform:methanol (2:1), the tubes were stirred for 5 min, and 2 phases were allowed to separate by low-speed centrifugation which leaves the denatured protein at the interphase. Under these extraction conditions, approximately 98% of Dol-P-Man is recovered in the lower organic phase, whereas Ret-P-Man partitions about 50:50 between the 2 phases (11). Alternatively, in Procedure 2, 15 volumes (3 ml) of chloroform:methanol (2:1, v/v) were added to yield a monophasic extract from which the denatured protein is removed by centrifugation.

Extracts were processed immediately. They were dried and immediately dissolved in suitable volumes of chloroform:methanol (2:1, v/v) containing 10 μg of synthetic Ret-P dissolved in methanol ready for application on thin layers of silica gel. Chromatography was usually performed in chloroform:methanol:water (45:35:6, by volume) (Solvent A) on 2 identical plates. One plate was used for radioactivity determination, and the other was used for fluorography unless stated otherwise. Sections of silica gel (0.5 cm) were collected into counting vials, and radioactivity was determined after addition of 0.25 ml of methanol and 10 ml of Betafluor (National Diagnostics, Somerville, N. J.). Fluorography was performed with Kodak film XR-5 as described above. Scraping and counting of radioactive spots were done after fluorography, when so specified. When exogenous Ret-P (10 μg) dissolved in methanol containing 30 mM ammonium acetate was included in the incubation mixture to study enzyme activity, MnCl₂ was used at 2.5 mM which gives maximum activity (11). Under these conditions of incubation, Ret-P-Man synthesis was monitored by the filtration assay as described elsewhere (10); about 90% of the radioactivity retained on the filter is Ret-P-Man. Alternatively, the biphasic extraction procedure was used, and the lower organic phase was utilized for measurement of radioactivity, 90% of which represents Ret-P-Man (11). Similar conditions were used for Dol-P-Man synthesis except that Dol-P (50 μg) was dissolved in 10 μl of 0.5% Triton X-100 (final detergent concentration, 0.025%), and the lower phase of the biphasic extraction procedure was used for measurement of radioactivity.

Other Procedures. Protein was determined by the method of Lowry et al. (14), with bovine serum albumin as a standard. Ret-P was measured spectrophotometrically on the basis of ε₃₂₅ (in methanol) = 1440. A recent report describes the physicochemical characteristics of Ret-P in detail (25).

RESULTS

Retinyl Palmitate Contents of Postnuclear Membranes from Hepatoma, Host Rat Liver, and Regenerating Liver. Table 1 shows that, in contrast to membranes from host liver tissue and regenerating liver, the membranes from hepatoma tissue contained less than detectable levels of retinyl palmitate. While trace
amounts (less than 5% of total vitamin A) of retinol and of other, more and less hydrophobic retinyl esters were detected by our procedures in the host liver tissue, the hepatoma tissue did not contain detectable amounts of these retinoids. In addition, the retinyl palmitate content of a primary cystic hepatocellular carcinoma induced by AAF was also determined. The primary cystic tumor was free of surrounding normal tissue and of detectable retinyl palmitate. The relatively wide variation in retinyl palmitate content of host liver is similar to that found in normal liver of several species.

**Synthesis of Ret-P-Man and Dol-P-Man from Endogenous Ret-P and Dol-P in Transplantable Liver Tumors.** The Morris hepatoma series varying in degree of differentiation and growth rate as described (10) was tested to study the relative proportion of Ret-P-Man to Dol-P-Man.

In most of these studies, a range of MnCl₂ concentrations was used, and the maximum synthesis for both mannolipids was usually at 5 mM, in agreement with published data (11). The results are expressed in the various charts as ratios of Ret-P-Man to Dol-P-Man obtained from the same incubation unless stated otherwise. Table 2 gives the actual amounts of radioactive mannose incorporated into Ret-P-Man and Dol-P-Man synthesized at 15 min as well as the ratios for the 5 mM MnCl₂ incubations.

**Morris Hepatoma 7777.** Fig. 1A shows the fluorograph of the mannolipids obtained in a monophasic extract at 0, 2.5, 5, 10, and 50 mM MnCl₂. The host liver shows prominent bands in the position of Ret-P-Man in the fluorograph of the tumor tissue (not shown). No prominent band was discernible in the position of undecaprenol phosphate mannose also found in other studies (11). Fig. 18 shows the ratio of Ret-P-Man to Dol-P-Man obtained from an identical TLC study of host liver and hepatoma tissue. The ratio in the latter tissue is about 10-fold less than in the host liver. The possibility that a specific decrease in enzyme activity might be responsible for a decrease in the Ret-P-Man:Dol-P-Man ratio was investigated. A 5-fold decrease in enzyme activity for Ret-P-Man synthesis was measured in the presence of excess Ret-P (135 μM) at 2.5 mM MnCl₂ as shown in Fig. 1C. However, a similar 5-fold decrease was also observed in the synthesis of Dol-P-Man in the presence of excess (135 μM) Dol-P (Fig. 1C). Thus, the difference in ratio does not seem to be due to differences in enzyme activity for Ret-P-Man and Dol-P-Man synthesis. Fig. 1D shows the fluorographs of a comparative TLC profile study between the host liver and tumor microsomal membranes incubated in the presence of 135 μM Ret-P.

**Morris Hepatoma 3924A.** Fig. 2, A and B, shows the fluorographs of the thin-layer chromatographs of host liver and tumor monophosphates extracts. In the host liver, the main radioactive spots correspond to Dol-P-Man, Ret-P-Man, mannose, and GDP-mannose (Fig. 2A). In the 3924A tumor (Fig. 2B), as for the 7777 (not shown), a prominent band ("Unknown") is found with intermediate migration between Ret-P-Man and Dol-P-Man. At 5 mM MnCl₂.

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**Table 1**

<table>
<thead>
<tr>
<th>Retinyl palmitate of hepatoma, host rat, and regenerating liver postnuclear membranes</th>
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| Tissue extracts were prepared from postnuclear membranes as specified under "Materials and Methods" and analyzed by reverse-phase high-pressure liquid chromatography on columns (4.6 mm (internal diameter) x 25 cm) of Partisil-10-ODS. Each liver extract was analyzed in triplicate (except for regenerating liver which was analyzed in duplicate). Regenerating liver tissue was analyzed at 24 and 48 hr after hepatectomy. The limit of detectability was 5 ng of retinyl palmitate. Each tumor extract was analyzed in duplicate except for 9618A-1-1, 44-2-1, and 5123tc, analyses of which were performed on 2 different tumors in duplicate for each. Invariably, retinyl palmitate and retinol were below detection limits in the tumors. In all cases, the equivalent of 7.28 mg of membrane protein was used, and the maximum synthesis for both mannolipids was usually at 5 mM MnCl₂, in agreement with published data (11). The areas corresponding to Ret-P-Man and Dol-P-Man were scraped from thin-layer chromatographs of host liver and tumor samples. In one experiment, the equivalent of 7.28 mg of membrane protein from 7777 tumor was injected, and 1.57 ng of retinyl palmitate per mg of protein could be measured.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Retinyl palmitate (ng/mg protein)</th>
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<tr>
<td>Liver</td>
<td>Host rat</td>
</tr>
<tr>
<td>20-1-1</td>
<td>580 ± 30</td>
</tr>
<tr>
<td>16-2-1</td>
<td>513 ± 52</td>
</tr>
<tr>
<td>7777-1-1</td>
<td>400 ± 21</td>
</tr>
<tr>
<td>9618A-1-1</td>
<td>942 ± 2.2</td>
</tr>
<tr>
<td>44-1-2</td>
<td>547 ± 21</td>
</tr>
<tr>
<td>5123D-1-1</td>
<td>150 ± 3.8</td>
</tr>
<tr>
<td>3924A-1-1</td>
<td>177 ± 59</td>
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<tr>
<td>7800-1-1</td>
<td>73 ± 1.6</td>
</tr>
<tr>
<td>5123tc-1-2</td>
<td>383 ± 6.7</td>
</tr>
<tr>
<td>7777-2-1</td>
<td>243 ± 38</td>
</tr>
<tr>
<td>Primary cystic tumor</td>
<td>302 ± 28</td>
</tr>
<tr>
<td>Regenerating liver (24 hr)</td>
<td>113 ± 7</td>
</tr>
<tr>
<td>Regenerating liver (48 hr)</td>
<td>100 ± 10</td>
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* Mean ± S.D.

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**Table 2**

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<th>Mankolipids of liver and hepatoma tissue</th>
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| Microsomal membranes (1 mg) were incubated as specified under "Materials and Methods" with 0.2 μCi of GDP-[14C]mannose (5 μM) in the presence of bovine serum albumin at 37°C for 15 min. The incubation mixture was then extracted with 15 volumes (3 ml) of chloroform:methanol (2:1, by volume) to obtain a monophasic extract which was processed as specified under "Materials and Methods" by TLC. The areas corresponding to Ret-P-Man and Dol-P-Man were scraped from thin-layer plates, and radioactivity was measured by liquid scintillation counting.

| Host liver | 1,900 | 5,800 | 0.30 |
| 20-1-1 host rat liver | 2,800 | 8,000 | 0.30 |
| 16-2-1 host rat liver | 450 | 6,800 | 0.07 |
| 9618A-1-1 host rat liver | 1,550 | 5,090 | 0.30 |
| 9618A-1-1 hepatoma | 990 | 10,000 | 0.10 |
| 44-1-2 host rat liver | 1,700 | 6,600 | 0.26 |
| 44-1-2 hepatoma | 300 | 8,700 | 0.03 |
| 5123D-1-2 host rat liver | 1,800 | 5,750 | 0.31 |
| 5123D-1-2 hepatoma | 400 | 10,000 | 0.04 |
| 3924A-1-1 host rat liver | 900 | 6,800 | 0.13 |
| 3924A-1-1 hepatoma | 270 | 4,800 | 0.05 |
| 7800-1-1 host rat liver | 1,870 | 5,280 | 0.35 |
| 7800-1-1 hepatoma | 190 | 2,380 | 0.07 |
| 5123tc-1-2 host rat liver | 950 | 4,000 | 0.23 |
| 5123tc-1-2 hepatoma | 350 | 8,700 | 0.05 |
| 7777-2-1 host rat liver | 1,120 | 5,580 | 0.20 |
| 7777-2-1 hepatoma | 80 | 3,440 | 0.02 |
| Guinea pig liver | 1,200 | 5,320 | 0.22 |
| Line 10 hepatoma | 1,000 | 3,600 | 0.03 |
| Normal hamster liver | 80 | 1,200 | 0.29 |
| Vitamin A-deficient hamster liver | 171 | 4,730 | 0.03 |

* In these samples, 0.5 μCi (12.5 μM) of GDP-[14C]mannose was used.
the ratio of Ret-P-Man to Dol-P-Man was decreased 2.9-fold in the tumor (Table 2). However, as for the 7777 membranes, no specific decrease in enzyme activity in the presence of exogenous Ret-P and Dol-P was found (not shown), thus again suggesting that a decrease in the endogenous pool of Ret-P is responsible for the lower ratio.

**Morris Hepatoma 5123tc-1-2, 5123D-1-2.** The ratios of Ret-P-Man to Dol-P-Man at various MnCl₂ concentrations compared to host liver microsomes are shown in Chart 1A for 5123tc and in Chart 1B for 5123D-1-2, showing a decrease in the tumor. A compound of intermediate migration between Ret-P-Man and Dol-P-Man ("Unknown") is found in 5123D-1-2 (not shown).

**Morris 7787 and 9618A-1-1.** The host liver pattern for 9618A-1-2 showed 2 major mannolipids comigrating with standard Ret-P-Man and Dol-P-Man (not shown). In 9618A membranes, the 2 major mannolipids comigrated with standard Ret-P-Man and Dol-P-Man (not shown). The Ret-P-Man:Dol-P-Man ratio was found to be decreased in Tumor 9618A compared to host liver from 0.3 to 0.1 at 5 mM MnCl₂ (Table 2). Chart 2A shows the Ret-P-Man:Dol-P-Man ratios for 9618A microsomes in incubations containing specified concentrations of MnCl₂. CRBP was measured by polyacrylamide gel electrophoresis of the cytosolic fractions after incubation with radioactive retinol as specified under Chart 2B. Host liver cytosol contained 4450 fmol/mg protein compared to 1030 fmol/mg protein for the 9618 hepatoma cytosol.

**Morris 16-2-1 and 20-1-1.** Fluorography of the thin-layer chromatographic profile of the monophasic extract of host liver and hepatoma 16-2-1 microsomes in duplicate was conducted as usual (not shown). The plates were scraped after chromatography, and the liver tissue showed a Ret-P-Man:Dol-P-Man ratio of about 0.3, while the tumor showed a ratio of 0.07. Similar reductions in ratio were obtained for the tumor 20-1-1 and for 44-2-1, as shown in Table 2.

**DMN-OAc-induced Hepatoma and Line 10 Hepatoma.** The fluorography of the thin-layer chromatographic profile of the lower organic phase extract of the DNA-OAc tumor postnuclear membranes at various MnCl₂ concentrations failed to show any Ret-P-Man after exposure of the film for 32 days. The ratio of Ret-P-Man to Dol-P-Man at the various MnCl₂ concentrations is shown in Chart 3, and it is decreased from 0.2 in the host liver to 0.02 for the tumor at 5 mM MnCl₂ (Table 2).

A comparison of mannolipid profiles between the line 10 guinea pig hepatoma and host liver postnuclear membranes (Fig. 3A) and the postnuclear membranes from vitamin A-normal and A-deficient hamster liver is shown (Fig. 3B). It becomes apparent that the patterns from the line 10 tumor and the deficient liver are very similar and that both show a decrease in Ret-P-Man and the prominence of new bands ("Unknown") of intermediate migration as indicated. The actual amounts of radioactivity found
as Ret-P-[14C]Man in the line 10 hepatoma and the host liver microsomes are shown in Fig. 3C.

AAF-induced Hepatoma and Host Liver Postnuclear Membranes. Fig. 4 shows a comparison of the TLC profiles of the radioactively labeled mannolipids synthesized in 15 min at 37° in host liver membranes (Fig. 4, Track A), primary tumor tissue (Fig. 4, Track B) of the cystic hepatocarcinoma type induced with AAF, and a solid hepatocarcinoma tumor tissue mixed with normal tissue (Fig. 4, Track C), all incubated with 5 mw MnCl2. It can be seen readily that the cystic hepatocarcinoma tumor (Fig. 4, Track B) synthesized very little Ret-P-Man and made a product of intermediate mobility ("Unknown") between Ret-P-Man and Dol-P-Man. Lane C indicates that tumor tissue did not contain activity to hydrolyze Ret-P-Man.

The actual amount of radioactivity was determined after scraping of the silica gel and liquid scintillation counting and showed a decrease in Ret-P-Man from 10,800 cpm in host liver microsomes to 1,200 cpm in the cystic tumor and 4,000 cpm in the solid tumor. Thus, compared to control, a reduction of 90% in Ret-P-Man was observed in the cystic tumor, whereas the mixed solid tumor had about 60% of the activity. Although the amount of Dol-P-Man synthesized was not determined in this experiment, no major decrease is visible in the tumor. It is of interest that the amount of retinyl palmitate was below detection in the cystic tumor (Table 1) and about one-half (160 ± 15 ng/mg protein) that of host liver tissue (302 ± 28 ng/mg protein) in the solid tumor mixed with surrounding tissue. When enzyme activities were compared in the presence of exogenous Ret-P and Dol-P, both tumors showed approximately one-half the accumulation of products (Ret-P-Man and Dol-P-Man) than did the normal tissue, but no specific significant differences were observed between Ret-P-Man and Dol-P-Man synthesis for the 2 tumors (not shown). Thus, the reduction in Ret-P-Man in Lane B is probably due to a specific decrease in endogenous Ret-P.

A study of regenerating liver (Fig. 5) at 24 hr after hepatectomy (Track C), of sham-operated (Track B) and of normal (Track A) liver demonstrates that partial hepatectomy (Track C; Table 2) does not decrease the amount of Ret-P-Man made. The amounts of radioactivity were determined after scraping and counting of Ret-P-Man and Dol-P-Man spots in Tracks A, B, and C. Radioactivity in Ret-P-Man was 3,500 cpm for normal rat liver tissue (Dol-P-Man, 8,000 cpm; ratio, 0.28), 4,900 cpm for sham-operated liver (Dol-P-Man, 17,500 cpm; ratio, 0.27), and 4,600 cpm for 24-hr-hepatectomized liver (Dol-P-Man, 16,700; ratio, 0.27).

Storage in liquid nitrogen for more than 1 month resulted in loss of Ret-P-Man and Dol-P-Man synthesis, whether from nor-
mal liver, host liver, or solid tumor mixed with normal liver (not shown). It also results in loss of enzyme activity with exogenous Ret-P and Dol-P to less than 20% of fresh liver preparation (not shown).

**DISCUSSION**

This work demonstrates that postnuclear membranes from hepatocellular carcinoma tissue of the Morris hepatoma series are in a status of relative depletion of vitamin A, compared to the membranes from liver of the host rat and from regenerating liver. The HPLC procedure used in these studies yields unequivocal results. It should be mentioned that attempts to measure vitamin A directly by UV absorption spectrometry of the chlo-roform:methanol extract of the membranes from tumor cells yielded false positives. The UV absorption at 325 nm found in an experiment with the 7777 tumor membranes was not due to either retinol or retinyl palmitate (results not shown), as found out by subsequent HPLC analysis of the extracts.

Therefore, it can be concluded from these studies that the storage function of liver for vitamin A has been lost in the tumor. This may simply be the reflection of tissue simplification due to loss during carcinogenesis of the fat-storing cells, which appear to contain about 80% of the retinyl palmitate of normal rat liver (5, 12). However, at least 7% (12) of the retinyl palmitate should be in parenchymal cells; therefore, if the hepatoma cells contained vitamin A similar to those in normal liver parenchyma (about 30 ng/mg of protein), our assay would have detected it. Instead, the highest measurable amount of vitamin A in 7777 hepatoma tissue was 1.57 ng/mg of membrane protein (5, 12). However, at least 7% (12) of the retinyl palmitate should be in parenchymal cells; therefore, if the hepatoma cells contained levels of vitamin A similar to those in normal liver parenchyma (about 30 ng/mg of protein), our assay would have detected it. Instead, the highest measurable amount of vitamin A in 7777 hepatoma tissue was 1.57 ng/mg of membrane protein (22). Although the concentration of vitamin A was not measured in the cells, it was assumed to be low in the absence of serum. Since the cells were responsive to the addition of retinol (0.35 or 3.5 nmol/ml), it must be assumed that they possess a mechanism for retinol uptake and therefore may not be vitamin A deficient when cultured in the presence of serum.

Recently (10), we have put forward the concept that the deficiency of an essential nutrient (e.g., vitamin A) or of its functions (e.g., the synthesis of a glycosylated cell surface receptor) could be involved in the promotion stage of carcinogenesis and may constitute a permissive condition for tumor development. The promoting stimulus may act by causing a "localized" deficiency status to which the normal (noninitiated) cell does not adapt but which the mutated cell may seize as a condition in which to express its tumorigenic phenotype. It is required by this concept that the essential function or nutrient normally antagonizes the expression of the tumorigenic phenotype in the initiated cell. It is of interest that deficiencies of other essential nutrients such as folic acid (9) and defects in vitamin B<sub>6</sub> metabolism (15) have also been reported to occur in neoplastic or preneoplastic tissue.

Finally, the present work offers a conceptual clue to explain the presence of altered carbohydrate chains in some neoplastic cells on the basis of a change in the makeup of lipid intermediate patterns. The accumulation of the new manno-olipoids represents a striking similarity between the cell membrane of some tumors and the membrane from vitamin A-deficient animals. Although the structure and function of these newly detected manno-olipoids are unknown, we may suggest that their presence in the membrane may be capable of altering the biosynthetic profile of vitamin A.
glycoproteins. Similarly, a decrease in the pool of Ret-P over Dol-P may also cause alterations on the final products of glycosylation and finally lead to an altered cell surface and secretory pattern and function. These possibilities are being probed experimentally.

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REFERENCES


Fig. 1. Synthesis of Ret-P-Man and Dol-P-Man in microsomes from host liver and Morris 7777 hepatoma. A, dependence of endogenous host liver microsomal mannolipid synthesis on Mn2+. Conditions were as described previously; GDP[14C]mannose (0.2 μCi; 5 μm) was used in each incubation. The monoclonal extraction procedure was utilized. The film was exposed for 4 weeks. Man, mannose, B, Ret-P-Man;Dol-P-Man ratios in host liver and Morris 7777 hepatoma microsomes. The actual amounts of radioactivity were obtained from plates run in duplicate to those used in fluorography. The silica gel was then scraped in 0.5-cm sections, and the radioactivity was determined. C, Ret-P-Man synthesis in Morris 7777 hepatoma and host liver microsomes from exogenous Ret-P and Dol-P. The incubation was the same as for the endogenous study. Protein (1 mg), 0.1 μCi of GDP[14C]mannose (24 μm), and 10 μg of Ret-P or 50 μg of Dol-P were used in each incubation. At appropriate times, the reaction was stopped with 1 ml of ice-cold 50 μm Tris-HCl (pH 7.4) containing 5 mM MgCl2, 1 mM KCl, and 0.25 mM sucrose (Medium A); the mixture was filtered on Millipore filters; the filters were washed with 1 ml of ice-cold Medium A, and radioactivity was determined in a Betalfluor for Ret-P-Man synthesis. The incubations used for Dol-P-Man synthesis contained 0.25% Triton X-100, in addition to the other components. The reaction was stopped by addition of 5 volumes of chloroform:methanol (2:1), and the lower organic phase was used for measurement of radioactive Dol-P-Man. D, fluorograph of TLC profile of Ret-P-Man synthesis from exogenous Ret-P in Morris 7777 hepatoma and host liver microsomes. Conditions of incubation were as described in C. The reaction was stopped with 5 volumes (1 ml) of chloroform:methanol (2:1) to yield the biophase extract. The lower organic phase was used for TLC in Solvent A as described under "Materials and Methods." Films were exposed for 3 days. Tracks marked St. contained standard Ret-P[14C]Man prepared from rat liver microsomes.

Fig. 2. A, fluorograph of TLC profile of mannolipids synthesized from endogenous lipid acceptors of host rat liver microsomes at various Mn2+ concentrations. Incubation contained 0.2 μCi of GDP[14C]mannose (5 μm) which was extracted to yield one phase. Tracks marked St. show the migration of standard Ret-P-Man;Dol-P-Man ratios in host liver microsomes. Unknown refers to a band of intermediate migration between Ret-P-Man and Dol-P-Man in the hepatoma tissue. Conditions of incubation were as described above.

Fig. 3. A, fluorography of the profiles of mannolipids from guinea pig (host) liver and 10 hepatoma postnuclear microsomes. Film was exposed for 4 weeks. Mannose, B, the same experiment as in A, conducted on postnuclear membranes from "normal" and vitamin A-depleted liver tissue. The lower (organic) phase of the biophase extraction procedure was used for the analysis in Solvent A. Film was exposed for 39 days. Tracks marked St. shows the behavior of a mixture of standard Ret-P-Man and Dol-P-Man. C, Ret-P-Man synthesis from endogenous Ret-P of host guinea pig liver (╳) and line 10 hepatoma (O) microsomes. Procedures were as described above.
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3A. Host Liver Line 10 Hepatoma

2.5 5 10

- Dol-P-Man
- Unknown
- Ret-P-Man
- Man
- GDP-Man

Normal

2.5 5 10

- Dol-P-Man
- Unknown
- Ret-P-Man
- Man
- GDP-Man

A-Deficient

2.5 5 10

- Dol-P-Man
- Unknown
- Ret-P-Man
- Man
- GDP-Man

2B.

St. 0 2.5 5 10 50 St.

[S_Cl2], mM

- Dol-P-Man
- Unknown
- Ret-P-Man
- Man
- GDP-Man

2C.

St. 0 2.5 5 10 50 St.

[S_Cl2], mM

- Dol-P-Man
- Unknown
- Ret-P-Man
- Man
- GDP-Man

3C.

[14C]-CPM/mg/15

0 100 200 300 400 500 600

0 2.5 5 10 50

[Fe2+] mM

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Retinyl Palmitate, Retinyl Phosphate, and Dolichyl Phosphate of Postnuclear Membrane Fraction from Hepatoma, Host Liver, and Regenerating Liver: Marginal Vitamin A Status of Hepatoma Tissue

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