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EFFECTS OF VITAMIN A DEFICIENCY ON THE LEVELS AND DISTRIBUTION OF RETINOL-BINDING PROTEIN AND MARKER ENZYMES IN HOMOGENATES AND GOLGI-RICH FRACTIONS OF RAT LIVER

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Summary

The levels of retinol-binding protein, prealbumin, and several 'marker' enzymes were determined in homogenates, crude subcellular fractions and isolated Golgi apparatus prepared from the livers of vitamin A-deficient and control rats. Vitamin A deficiency led to a marked increase (3.5-fold) in hepatic retinol-binding protein concentration and to slight increases in hepatic prealbumin levels, without affecting the levels of a number of marker enzymes localized in various subcellular compartments. The distributions of total protein and marker enzymes among various subcellular fractions were nearly identical in control and vitamin A-deficient preparations. In particular, vitamin A deficiency had no effect on the yield or enzymatic composition of isolated Golgi-rich fractions. In vitamin A-deficient rats, where the normal secretion of retinol-binding protein was blocked, a maximum of less than 10% of the total liver retinol-binding protein was accounted for in the Golgi. In contrast, in control rats, where the secretion of retinol-binding protein was proceeding at the normal rate, the relative amount of retinol-binding protein in Golgi increased to about 23% of the total liver pool. The data suggest that the Golgi apparatus is involved in the pathway of retinol-binding protein secretion from the liver, but demonstrate that the Golgi is not the major subcellular locus for retinol-binding protein in either normal or vitamin A-deficient rats.

Introduction

Vitamin A is transported in plasma bound to its specific transport protein,

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plasma retinol-binding protein [1,2]. In plasma, retinol-binding protein circulates mainly as a 1 : 1 molar protein-protein complex with another protein, prealbumin. Both of these proteins are synthesized and secreted by the liver.

Studies in the rat have demonstrated that vitamin A deficiency primarily interferes with the secretion, rather than the synthesis, of retinol-binding protein by the liver and that the deficient liver contains an expanded pool of preformed apo-retinol-binding protein that can be released rapidly into the serum when vitamin A becomes available [2—4]. In contrast, vitamin A deficiency has little effect on the liver and serum levels of prealbumin [5], and it has been concluded that the synthesis and secretion of retinol-binding protein and prealbumin are regulated by independent mechanisms.

The intracellular events leading to the production and secretion of retinol-binding protein by the liver and the manner in which vitamin A deficiency interferes with these processes are largely unknown. Liver retinol-binding protein is membrane bound and is recovered mainly in the microsomal fraction of liver homogenates from both normal and vitamin A-deficient rats [6]. It has also recently been demonstrated that colchicine, a drug known to interfere with cellular microtubule function, inhibits the normal and retinol-stimulated secretion of retinol-binding protein by the liver [7].

Little systematic information is available on the effects of vitamin A deficiency on the subcellular organization and composition of rat liver. Hence, the present studies were developed in order to explore the effects of vitamin A deficiency on the levels and subcellular distributions of a number of hepatic constituents. Furthermore, since the Golgi apparatus is importantly involved in the secretion of plasma proteins by the hepatocyte [8—11], we sought to obtain quantitative information on the role of the Golgi apparatus in retinol-binding protein secretion using both normal and vitamin A-deficient rats.

Experimental procedure

Animals and dietary treatments. Twelve male, weanling rats were obtained from the Holtzmann Company (Madison, WI) and fed (ad libitum) purified diets prepared in this laboratory as described previously [3,4]. Six rats were depleted of vitamin A by feeding them a vitamin A-deficient diet for four weeks; thereafter, these rats were fed the same diet supplemented with retinoic acid (12 μg/g diet) in order to maintain good growth and general health. The retinoic acid supplementation was discontinued one week before killing. Six rats which served as controls were fed the basal vitamin A-free diet supplemented with vitamin A (4.5 μg retinol equiv./g diet as retinyl esters) from weaning onwards. The rats used for these experiments were adults of approx. 250 g body weight.

Preparation of crude subcellular fractions and Golgi-rich fractions. The six control and six vitamin A-deficient rats were killed by decapitation and the livers were removed, weighed and kept at 4°C for all subsequent procedures. Samples of blood for the preparation of sera were also obtained from each rat. Table I shows the liver weights and retinol-binding protein levels in the livers and sera of the rats used in these experiments. Livers were minced in pairs (see Table I) by passage through a tissue press, to yield three pools of control liver

TABLE I

LIVER WEIGHTS AND RETINOL-BINDING PROTEIN (RBP) CONCENTRATIONS IN LIVER AND SERUM OF CONTROL AND VITAMIN A-DEFICIENT RATS

Values for liver RBP were determined for liver homogenates prepared from pairs of control (A—C) or deficient (D—F) animals.

	Liver weight (g)	Serum RBP (μg/ml)	Liver RBP (μg/g)
Control rats			
A	13.0	60.3	24.2
A_1	11.8	59.0	
B	12.0	49.3	25.8
B_1	10.3	57.1	
C	12.3	49.4	22.4
C_1	10.2	52.4	
Deficient rats			
D	10.5	12.2	72.2
D_1	8.3	8.2	
E	8.8	9.1	76.2
E_1	8.5	9.9	
F	7.7	6.9	85.4
F_1	9.1	8.4	

tissue and three pools of deficient material. The individual pools were then fractionated as described below.

For the preparation of a Golgi-rich fraction, 13-g portions of the minced liver (from each of the six pools) were homogenized and subjected to combined differential and density gradient centrifugation as described by Morré [12]. Portions of the original homogenates and the combined supernatants from all steps in the isolation were saved in order to assess the total recoveries of all constituents.

For the preparation of crude subcellular fractions, 4 g of each mince were mixed with 9 vols. of a solution comprising 0.25 M sucrose, 0.05 M Tris-HCl, pH 7.6, 0.025 M KCl, and 0.005 M $MgCl_2$ and homogenized with ten strokes of a Potter-Elvehjem homogenizer. Portions of the resulting homogenate were fractionated by successive centrifugation at $400 \times g$ for 10 min, $10\,000 \times g$ for 10 min, and $105\,000 \times g$ for 1 h to yield crude nuclear, mitochondrial, and microsomal fractions and the soluble fraction.

All fractions were assayed shortly after preparation for NADPH-cytochrome *c* reductase, glucose-6-phosphatase, and (for the Golgi isolation) cytochrome oxidase. Other portions were frozen for subsequent assay of protein, acid phosphatase, glycosyltransferases, retinol-binding protein and prealbumin.

Assays for marker enzymes. The following enzymes were used as markers for the indicated subcellular components: cytochrome oxidase (mitochondria); glucose-6-phosphatase and NADPH-cytochrome *c* reductase (endoplasmic reticulum); acid β-glycerophosphatase (lysosomes), and three glycoprotein : glycosyltransferases (Golgi). Cytochrome oxidase [13], glucose-6-phosphatase [12], and NADPH-cytochrome *c* reductase [14] were assayed according to the

indicated published procedures. Acid phosphatase was assayed essentially as described by Glaumann and Dallner [14] except that Triton X-100 was not included in the reaction mixture. In order to release the full activity of this latent enzyme, all fractions were frozen and thawed three times prior to assay.

For determination of glycoprotein : glycosyltransferases, the transfer of radioactive sugars from labeled nucleotide sugars to exogenous glycoprotein acceptors was assayed in a manner similar to that described by Grimes [15]. The following nucleotide sugars were purchased from New England Nuclear (Boston, MA): UDPgalactose (D-[U-^{14}C]galactose), 257—281 Ci/mol; CMPsialic acid ([4,5,6,7,8,9-^{14}C] sialic acid), 217 Ci/mol; and UDP-*N*-acetylglucosamine (*N*-acetyl-D-[1-^{14}C]glucosamine), 56.5 Ci/mol. Sialic acid was removed from bovine submaxillary mucin (Sigma) by mild acid hydrolysis (0.1 N H_2SO_4, 85°C, 1 h). A number of pilot experiments were conducted to determine the optimal conditions for the assay of each of the three Golgi glycosyltransferases [16]. All assays were performed in a final volume of 0.1 ml and contained 0.1% Triton X-100. For galactosyltransferase the reaction mixture contained 25 mM Tris/maleate, pH 6.4, 10 mM $MnCl_2$, 2 μM UDPgalactose, and 300 μg of desialysed bovine submaxillary mucin. For sialyltransferase the reaction mixture contained 25 mM Tris/maleate, pH 6.8, 12.5 mM $MgCl_2$, 2.3 μM CMPsialic acid and 200 μg of desialysed bovine submaxillary mucin. For *N*-acetylglucosaminyl transferase the reaction mixture contained 25 mM Tris/maleate, pH 6.8, 10 mM $MnCl_2$, 4.4 μM UDP-*N*-acetylglucosamine, and 470 μg of native bovine submaxillary mucin. In each assay duplicate tubes with exogenous acceptor were assayed along with an identical tube without added acceptor in order to correct for endogenous transferase activity. For all three enzymes, the activity in the absence of added acceptor was less than 10% of that in the presence of exogenous glycoprotein.

All marker enzyme assays were conducted under conditions where product formation was proportional to the time of incubation and to the quantity of protein (enzyme) in the incubation mixture; in most cases three protein concentrations were used for each determination.

Other assays. Protein was determined by the method of Lowry et al. [17] using bovine serum albumin as a standard. Retinol-binding protein and prealbumin concentrations were determined by radioimmunoassay as previously described [5,6].

Statistical treatment. The statistical significance of differences in means was examined using the two-tailed Student's *t*-test [18] on a Wang 700 A programmable calculator. Differences between means were not considered to be significant when $P > 0.05$.

Results

Activities in whole homogenates

The concentrations of marker enzymes and of retinol-binding protein and prealbumin in whole rat liver homogenates are given in Table II. The activities of the marker enzymes studied were not significantly different in homogenates prepared from the livers of vitamin A-deficient as compared to control animals. In fact, the only constituents studied which were affected by vitamin A defi-

TABLE II

CONCENTRATION OF CONSTITUENTS IN WHOLE LIVER HOMOGENATES FROM CONTROL AND VITAMIN A-DEFICIENT RATS

Values are means ± 1 S.D. for two determinations each for three control and three deficient preparations. Units are defined as follows: cytochrome oxidase activity is given as the first order rate constant, k (min^{-1}), for the oxidation of cytochrome c [13]; NADPH-cytochrome c reductase, ΔA_{550} ($\times 10^2$)/min; glucose-6-phosphatase and acid phosphatase, μmol phosphate/h; retinol-binding protein and prealbumin, μg; sialyl and *N*-acetylglucosaminyltransferase, cpm of glycose transferred ($\times 10^{-3}$)/40 min; galactosyltransferase, cpm of glycose transferred ($\times 10^{-3}$)/20 min. Concentration of protein was 247 ± 29 mg/g liver for controls and 229 ± 33 mg/g liver for deficients.

Constituent	Units/mg protein	
	Control	Deficient
Cytochrome oxidase	3.40 ± 0.44	3.41 ± 0.41
NADPH-cytochrome *c* reductase	4.24 ± 0.51	4.23 ± 0.93
Glucose-6-phosphatase	1.20 ± 0.12	1.33 ± 0.14
Acid phosphatase	0.60 ± 0.13	0.74 ± 0.10
Prealbumin	0.24 ± 0.05	0.37 ± 0.05 *
Retinol-binding protein	0.10 ± 0.01	0.34 ± 0.03 **
Sialyltransferase	8.87 ± 2.40	11.03 ± 2.84
Galactosyltransferase	34.48 ± 12.51	47.41 ± 9.72
N-Acetylglucosaminyltransferase	1.42 ± 0.81	1.83 ± 1.04

* Significantly different from control with $P < 0.05$.
** Significantly different from control with $P < 0.001$.

ciency were the proteins involved in the serum transport of vitamin A. The concentration of retinol-binding protein was approximately 3.5 times greater in deficient preparations than in control homogenates. Hepatic prealbumin concentrations were about 1.5 times higher in deficient rats than in controls. Thus a deficiency of retinol specifically affected the hepatic levels of its serum binding protein without affecting a variety of other enzyme proteins localized in a number of subcellular compartments.

Fractionation by differential centrifugation

Rat liver homogenates were fractionated by differential centrifugation and the resulting fractions and the homogenates were assayed for retinol-binding protein, prealbumin, and for a number of marker enzymes. The marker enzymes assayed were NADPH-cytochrome *c* reductase and glucose-6-phosphatase (endoplasmic reticulum), acid phosphatase (lysosomes), and sialyl-, galactosyl-, and *N*-acetylglucosaminyltransferase (Golgi apparatus). In this experiment the particulate fractions were not washed by resuspension and recentrifugation and hence only a moderate and incomplete resolution of the various organelles was obtained (data not shown). However, as expected, the greatest enrichment of the two endoplasmic reticulum markers was found in the microsomal fraction, and that of the lysosomal marker in the mitochondrial fraction. Under these conditions, the three glycosyltransferases distributed identically with each other and were mainly enriched in the mitochondrial fraction.

The homogenates derived from normal and vitamin A-deficient livers were compared with regard to the distribution of total protein, retinol-binding protein, prealbumin, and of the various marker enzymes among the four crude sub-

TABLE III

RELATIVE SPECIFIC ACTIVITIES OF CONSTITUENTS IN GOLGI APPARATUS FROM THE LIVERS OF CONTROL AND VITAMIN A-DEFICIENT RATS

Relative specific activity = units/mg protein Golgi divided by units/mg protein in homogenate. Values are means ± 1 S.D., $n = 3$. The average percentage recovery (i.e., amount recovered in all fractions relative to the unfractionated homogenate) for each constituent was: protein, 105; cytochrome oxidase, 96; NADPH-cytochrome *c* reductase, 107; glucose-6-phosphatase, 114; acid phosphatase, 119; prealbumin, 84; retinol-binding protein, 108; sialyltransferase, 98; galactosyltransferase, 101, and *N*-acetylglucosaminyltransferase, 153.

Constituent	Relative specific activity in Golgi	
	Control	Deficient
Cytochrome oxidase	0.61 ± 0.14	0.44 ± 0.09
NADPH-cytochrome *c* reductase	2.63 ± 0.33	1.91 ± 0.32
Glucose-6-phosphatase	2.58 ± 2.05	1.70 ± 0.62
Acid phosphatase	5.58 ± 3.13	6.73 ± 0.96
Prealbumin	5.99 ± 1.55	5.27 ± 0.90
Retinol-binding protein	9.12 ± 2.00	3.65 ± 0.48 *
Sialyltransferase	43.21 ± 13.08	46.02 ± 11.70
Galactosyltransferase	39.57 ± 14.79	37.22 ± 9.76
N-Acetylglucosaminyltransferase	48.84 ± 12.74	69.36 ± 28.73

* Significantly different from control with $P < 0.01$.

cellular fractions. The distributions of all of the various constituents among the four subcellular fractions were nearly identical in the control and the vitamin A-deficient preparations.

Isolation of Golgi apparatus

A Golgi-rich fraction was isolated from homogenates of the livers of control and vitamin A-deficient rats as described above. 10—15 mg of Golgi protein was obtained from 13 g wet weight of liver; this represented approx. 0.45% of the total homogenate protein. Similar recoveries of Golgi protein were obtained with control and vitamin A-deficient preparations. The enzymatic compositions and the retinol-binding protein and prealbumin contents of these fractions are summarized in Table III. The isolated fractions were highly enriched in Golgi apparatus as assessed by the high relative specific activities (greater than 40) of the glycosyltransferases. The fractions were only slightly contaminated with microsomal and mitochondrial elements as indicated by the observed relative specific activities of the marker enzymes for these components. The level of lysosomal contamination was about the same as that reported by others [19]. As shown in Table III, no differences were observed between Golgi preparations isolated from normal or from vitamin A-deficient rats with regard to the relative specific activities of glycosyltransferases, of other marker enzymes, and of prealbumin.

Although vitamin A deficiency had no effect on the yield of Golgi apparatus or its enzymatic composition, the Golgi isolated from deficient rats showed a 2.5-fold decrease in the relative specific activity of retinol-binding protein when compared to controls. Thus the amount of retinol-binding protein in Golgi relative to the total liver pool was much less in deficient animals than in

controls. The maximal percentage of the total liver retinol-binding protein in Golgi can, in fact, be calculated assuming that: (1) the retinol-binding protein in the isolated Golgi fraction is entirely associated with Golgi-derived material, and (2) the glycosyltransferases are exclusively localized in Golgi. The value is given by:

$$\%\ \text{of liver retinol-binding protein in Golgi} = \%\ \text{retinol-binding protein recovered in Golgi} \times \frac{100}{\%\ \text{of glycosyltransferase activity recovered in Golgi}}$$

Applying this equation, we have calculated that the Golgi apparatus contained a maximum of about 23% of the total liver retinol-binding protein in control rats and of about 9% in vitamin A-deficient rats. Similar calculations for liver prealbumin yield values of 15% and 13%, respectively, for the Golgi content of prealbumin in control and vitamin A-deficient rats.

Discussion

The studies reported here provide information on the effect of vitamin A deficiency on the concentration and the subcellular distribution of a number of enzymes and proteins in rat liver. In agreement with previous reports from this laboratory [3—5], vitamin A deficiency led to marked increases in the levels of retinol-binding protein in the liver, and to slight increases in the concentration of prealbumin. In contrast, the levels of a number of hepatic marker enzymes, localized in a variety of subcellular organelles, were not affected by vitamin A deficiency (Table II). These findings strongly support the contention that a deficiency of retinol specifically affects the hepatic metabolism of its plasma transport protein.

The rats used in these studies were first depleted of their vitamin A stores, and were then maintained on the same diet supplemented with retinoic acid, in order to maintain growth and health. 1 week prior to experimentation they were again fed a diet totally devoid of any vitamin A. Thus, the effects observed here can be attributed solely to a specific deficiency of vitamin A and not to poor health often associated with long-term vitamin A deficiency.

In addition to having no effect on the absolute activities of the enzymes studied here, vitamin A deficiency had no effect on their distribution among crude subcellular fractions. As pointed out in Results, the subcellular fractionation experiment reported here was a crude one, and the organelles were not well separated from each other. Because the primary focus of the present study was on the Golgi apparatus, we carried out only a rapid and relatively crude overall differential centrifugation study to look for major differences in enzyme distribution resulting from vitamin A deficiency. No such differences were observed. The absence of such differences, coupled with the precise data reported on the Golgi apparatus, and with the results of more extensive fractionation work on vitamin A-deficient rat liver reported elsewhere [16,20], indicates clearly that vitamin A deficiency does not affect the major characteristics of the subcellular organelles of rat liver. Thus, vitamin A deficiency does not appear to alter the relative amount, the enzymatic composition, or the

general sedimentation properties of any of the major subcellular organelles of rat liver. The results demonstrate further the specificity of the effect of vitamin A deficiency on retinol-binding protein metabolism in the liver, and also indicate that in future studies the known techniques of subcellular fractionation can be used unambiguously to explore hepatic metabolism and function in the vitamin A-deficient rat.

Preliminary experiments from this laboratory [6] showed that fractions enriched in Golgi apparatus did contain retinol-binding protein, but suggested that the Golgi was not the major subcellular location of retinol-binding protein in either normal or vitamin A-deficient rats. Others have suggested, however, on the basis of retinol-binding protein analyses of not well-characterized Golgi fractions, that the Golgi may be a major site of retinol-binding localization in the liver [21]. The results reported here provide more fully quantitative data on the levels of retinol-binding protein in rat liver Golgi.

Both in terms of yield and enzymatic composition, the Golgi-rich fractions isolated from the livers of vitamin A-deficient rats were virtually identical to those from control rats. In contrast, the isolation of rat liver Golgi apparatus has been shown to be extremely sensitive to certain other dietary manipulations [22,23]. The lack of an effect of vitamin A deficiency on Golgi glycosyltransferases was also of interest, in view of the proposed role of retinol as a lipid intermediate in the mannosylation of membrane glycoproteins [24]. Although our results do not bear directly on this latter question, they do suggest that vitamin A deficiency does not affect the terminal glycosylation of a wide variety of proteins since there was no alteration in the activities involved in the addition of *N*-acetylglucosamine, galactose, or sialic acid to the termini of carbohydrate side chains.

Significant differences were found in the relative quantity of retinol-binding protein in Golgi in normal and vitamin A-deficient livers. In vitamin A-deficient rats, where the normal secretion of retinol-binding protein was blocked, a maximum of 9% of the total hepatic retinol-binding protein was found in the Golgi. In contrast, in normal rats where retinol-binding protein secretion was proceeding at a normal rate, a maximum of 23% of the total retinol-binding protein pool in the liver was found associated with the Golgi. The data clearly indicate that the Golgi apparatus is not the site of a large proportion of retinol-binding protein in the liver in either the normal or the vitamin A-deficient rat. This conclusion is consistent with other evidence that the Golgi apparatus is a transitory processing locus for secretory proteins and not a major intracellular storage site [8]. The data are also consistent with the suggestion that the Golgi apparatus is involved in the normal pathway of retinol-binding protein secretion and that the block in retinol-binding protein secretion found in vitamin A deficiency occurs at a site before the retinol-binding protein molecule reaches the Golgi. With the information available, however, it is not yet possible to define the site of blockage of retinol-binding protein secretion observed in vitamin A deficiency, or the mechanism by which retinol stimulates the secretion of retinol-binding protein from the liver cell. Studies aimed at elucidating these questions are currently in progress in this laboratory.

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