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ASCORBIC ACID DEFICIENCY AND HEPATIC UDP-GLUCURONYL TRANSFERASE

QUALITATIVE AND QUANTITATIVE DIFFERENCES

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Abstract—The effect of dietary ascorbate on hepatic UDP glucuronyltransferase (UDPGT) appears to be selective in that only certain isozymes of UDPGT are jeopardized. In this study, ascorbic acid deficiency produced a 68% reduction in the specific activity of hepatic UDPGT towards *p*-nitrophenol. Earlier studies showed a reduction in UDPGT activity towards *p*-aminophenol in ascorbate-deficient guinea pigs, whereas bilirubin and acetaminophen glucuronidation were unaffected. Kinetic studies suggest that *p*-aminophenol and *p*-nitrophenol are metabolized by a single isozyme in that *p*-nitrophenol was found to be a competitive inhibitor of *p*-aminophenol glucuronidation. Both qualitative and quantitative studies on partially purified UDPGT from ascorbate-deficient and ascorbate-supplemented guinea pigs were carried out to investigate the biochemical role of the vitamin. Qualitative differences were observed in UDPGT from ascorbate-deficient animals and included an increased lability to: thermal inactivation; storage at 4°; and purification with UDP-glucuronic acid agarose column chromatography. Furthermore, an analysis of the microsomal membrane showed a 14% increase in membrane fluidity in ascorbate deficiency. Ascorbic acid added *in vitro* could not reverse the increase in fluidity observed in ascorbate-deficient microsomal membranes; however, ascorbylpalmitate, a more lipophilic form of the vitamin, was effective. Palmitic acid had no effect on membrane fluidity in microsomes from either the ascorbate-supplemented or ascorbate-deficient animals. This increase in membrane fluidity could not be explained by differences in cholesterol, total phospholipid, or phosphatidylcholine content of hepatic microsomes. Furthermore, a quantitative reduction in UDPGT partially purified from ascorbate-deficient guinea pigs was indicated by a marked reduction in protein banding at 55,000 daltons when compared to UDPGT partially purified from ascorbate-supplemented animals.

UDP-glucuronyltransferase (UDPGT, EC 2.4.1.17) is quantitatively the most important detoxifying reaction in mammalian metabolism [1]. Multiple isozymes of UDPGT have been identified, each consisting of a different number of monomeric polypeptide chains [2, 3]. UDPGT is a tightly-bound microsomal enzyme and its activity is dependent on the membrane environment [4–7]. Factors which influence the membrane environment have been shown to alter UDPGT activity [4–12]. With respect to diet, protein restriction [8, 9], fat deprivation [10], cholesterol fatty liver [11], and choline deficiency [12] have all been shown to modify properties of the microsomal membrane and alter UDPGT activity. A deficiency in dietary ascorbic acid has also been shown to result in a reduction in the specific activity of UDPGT towards *p*-aminophenol [13]. There was no reduction in the specific activity towards acetaminophen or bilirubin, suggesting that one isozyme may be altered in ascorbate deficiency [13]. This reduction in specific activity of UDPGT in ascorbate-deficient guinea pigs was not due to an alteration in the apparent affinity either for the substrate, *p*-aminophenol, or the cofactor, uridine 5′-diphosphoglucuronic acid.

The purpose of this study was to investigate the biochemical role by which dietary ascorbic acid influences hepatic UDPGT. Qualitative and quantitative studies were carried out in partially purified UDPGT fractions from ascorbate-supplemented and ascorbate-deficient guinea pigs using the substrate *p*-nitrophenol. Studies examining qualitative properties of UDPGT included: thermal inactivation, stability of DEAE-cellulose fractions to storage at 4°, and purification characteristics. Several important properties of microsomal membranes from ascorbate-supplemented and ascorbate-deficient guinea pigs were also analyzed including membrane fluidity using an apolar core probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), and content of phosphatidylcholine, total phospholipid and cholesterol.

In addition, quantitative analysis of UDPGT protein involving subjection of partially purified UDPGT from ascorbate-supplemented and ascorbate-deficient animals to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out.

MATERIALS AND METHODS

Materials. Emulgen 911 was purchased from the Kao Co. (Tokyo, Japan). Triton X-100, sodium ascorbate, ascorbylpalmitate, UDP-glucuronic acid, UDP-*N*-acetylglucosamine, DEAE-cellulose, DEAE-Sephadex, horseradish peroxidase (80 units/mg protein), *p*-hydroxyphenylacetic acid, cholesterol ester-

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ase (bovine pancrease, 650 units/g protein), cholesterol oxidase (Pseudomonas, 40 units/mg protein), cholesterol (99%), L-phosphatidylcholine (egg yolk and bovine brain), UDP-glucuronic acid agarose, and DPH were purchased from the Sigma Chemical Co. (St Louis, MO). Sodium cholate was purchased from Fischer Chemicals (Fair Lawn, NJ). *p*-Nitrophenol was purchased from Eastman Kodak (Rochester, NY). Acrylamide and *N'*,*N'*-methylene bisacrylamide were purchased from International Biotechnologies, Inc. (New Haven, CT). Sodium dodecyl sulfate was purchased from BDH Chemicals Ltd (Poole, U.K.). Silica gel TLC plates (60 HTLC10 × 20 cm) were purchased from the Whatman Co. (Clifton, NJ). Ascorbic acid deficient diet (guinea pig pelleted) was obtained from Nutritional Biochemicals (Cleveland, OH). Male Hartley guinea pigs were purchased from the Michigan Department of Public Health (Lansing, MI). All chemicals used were of reagent grade.

Guinea pigs. Guinea pigs (young adults) were kept on the ascorbic acid-deficient diet *ad lib*. for 18–21 days; one group received 2 mg ascorbate/mL of drinking water daily and the other group received no ascorbic acid in their drinking water. Guinea pigs on the ascorbic acid-deficient regimen had minimal joint hemorrhages and were not frankly scorbutic. There were no significant differences in their rate of weight gain during the experimental period, compared with animals on the ascorbic acid-supplemented diet. The average weight of guinea pigs on the ascorbic acid-deficient diet was 360 ± 25 g while the average weight of the ascorbic acid-supplemented guinea pigs was 373 ± 14 g. Liver ascorbic acid content was 31.3 ± 5.7 mg/100 g and 3.7 ± 1.4 mg/100 g wet liver weight for the ascorbate-supplemented and -deficient animals respectively.

Preparation of guinea pig tissues. Animals were decapitated and exsanguinated, and their livers were quickly removed and placed on ice. All of the following procedures were carried out at 4°. Homogenates (20%, w/v) were prepared in 0.1 M sodium phosphate, pH 7.4, with a Potter–Elvehjem glass homogenizer. The homogenates were centrifuged at 12,000 *g* for 30 min. The 12,000 *g* supernatant fraction was centrifuged for 60 min at 100,000 *g* to harvest microsomes. The 12,000 *g* supernatant fraction and 100,000 *g* microsomal pellet were stored at −20°.

Ascorbic acid and protein determination. Ascorbic acid concentration was determined by the method of Zannoni *et al.* [14]. Protein was determined by the method of Lowry *et al.* [15] with bovine serum albumin as the standard.

Cytochrome P-450. Cytochrome P-450 was determined in the 100,000 *g* microsomal pellet from the reduced carbon monoxide difference spectrum according to the method of Omura and Sato [16] with assay conditions as previously described [13]. A molar extinction coefficient of 91 $mM^{-1}\ cm^{-1}$ was used.

Purification of UDP-glucuronyltransferase. The purification procedure was as described by Singh *et al.* [4] with modifications: Microsomes (250–300 mg protein/mL) in 25 mM Tris–HCl, pH 7.4, 25% glycerol, and 0.1 mM dithiothreitol (DTT) were solubilized with the addition of Emulgen 911 (0.67 mg detergent/mg protein). After stirring for 1 hr at 4°, the Emulgen 911-treated microsomes were centrifuged for 15 min at 100,000 *g* to obtain the supernatant fraction which contained 90% of the UDPGT activity. Approximately 10 mL of this fraction (15–18 mg protein/mL) was applied directly to a column of DEAE-cellulose (22 cm × 1 cm) which had been equilibrated previously with 25 mM Tris–HCl, pH 7.4, 25% glycerol, and 0.1 mM DTT. UDPGT protein was not retained and was eluted in the first major protein peak with 25 mM Tris–HCl, pH 7.4, 25% glycerol, and 0.1 mM DTT. The DEAE-cellulose eluant (4.0 mg protein/mL) was applied to a DEAE-Sephadex column (22 cm × 1 cm), previously equilibrated with 25 mM Tris–HCl, pH 7.4, 25% glycerol, and 0.1 mM DTT. UDPGT protein was not retained on this column and was eluted in the first major protein peak with 25 mM Tris–HCl, pH 7.4, 25% glycerol, and 0.1 mM DTT. The eluant from the DEAE-Sephadex column (1.0 to 2.0 mg protein/mL) was then applied to a high affinity UDP-glucuronic acid agarose column (6 cm × 0.5 cm) previously equilibrated with 25 mM Tris–HCl, pH 7.4, 25% glycerol, and 0.1 mM DTT. The column was washed with a 2-bed volume of 25 mM Tris–HCl, pH 7.4, 25% glycerol, and 0.1 mM DTT, and UDPGT was eluted with a 2-bed volume of 5 mM UDP-glucuronic acid.

p-*Nitrophenol glucuronidation*. The assay was as described by Isselbacher *et al.* with modifications [17]: Microsomes (20–25 mg protein/mL) were treated with Emulgen 911 (0.67 mg detergent/mg protein) with continuous stirring at 4° for 30 min to activate UDPGT. The reaction mixture contained: 100 mM sodium phosphate buffer, pH 7.1, 1 mM UDPGA, and 0.6 mM *p*-nitrophenol in a final volume of 0.4 mL; incubations were carried out in 10-mL Erylenmeyer flasks at 37°. Aliquots (0.75 μL) were removed at 0, 5, 10, and 20 min and placed in 1.5 mL of 0.25 N KOH. The samples were spun at 10,000 *g* for 5 min, and the *p*-nitrophenol glucuronidation was measured spectrophotometrically by the absorbance decrease at 400 nm. A molar extinction coefficient of 1840 $M^{-1}\ cm^{-1}$ was used.

UDP-N-*acetylglucosamine: specific activator*. Assay conditions were as described above except that hepatic microsomes were preincubated with UDP-*N*-acetylglucosamine (2.0 mM) for 15 min, and *p*-nitrophenol activity was assayed as described above.

Flavin monooxygenase activity towards dimethylaniline. Dimethylaniline metabolism was measured by following the oxidation of NADPH at 340 nm as described by Tynes and Hodgson [18] with modifications described by Brodfuehrer and Zannoni [19]. The reaction rates were determined with a molar absorption coefficient of 6220 $M^{-1}\ cm^{-1}$.

Preparation of L-*phosphatidylcholine micelles*. Phosphatidylcholine micelles were prepared as previously described [13]. Phosphatidylcholine [0–25 μg P (phosphorus)/mg protein] was preincubated with UDPGT for 30 min on ice before assaying for *p*-nitrophenol activity as described above.

Measurement of total phospholipids. Total phospholipids were measured by the method of Raheja *et al.* [20]. Lipids were extracted from crude hepatic microsomes (0.3 mL of 20 mg protein/mL) with 4.5 mL of chloroform/methanol (2:1). The extracted lipids (175 μL) were dried in a heating block

(60°) under a stream of nitrogen. Chloroform (0.4 mL) and 0.1 mL of a chromogenic solution were added to the dried lipid. The chromogenic solution reacts directly with the phospholipid phosphorus and a Prussian blue complex is formed which has an absorption maximum at 710 nm. Phosphatidylcholine (0 to 4.1 μg P/mg) from bovine brain (10 mg protein/mL) was used as the standard.

TLC spot analysis of phosphatidylcholine. The specific quantity of phosphatidylcholine was determined as described by Levy *et al.* [21] with modifications: phospholipids from hepatic microsomes were extracted with chloroform/methanol (2:1) and dried on a heating block (60°) under a stream of nitrogen as described above. The dried lipid was extracted with 100 μL of chloroform/methanol (2:1), vortexed for 1 min, and analyzed by thin-layer chromatography. Samples were spotted with a 10 μL Hamilton syringe on a HPTLC 60 plate previously treated with methanol. The samples were washed with 30 and 20 μL of chloroform/methanol (2:1), vortexed for 30 sec, and spotted on the plates. The plates were chromatographed in chloroform/methanol/acetic acid/distilled water (100:30:35:3, by vol.). The solvent system separates phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and phosphatidylcholine with R_f values of: 0.60, 0.44, 0.33 and 0.17 respectively. After development, the plates were dried and phospholipid spots were visualized by iodine vapor and identified by comparison to standards (Sigma). The spots representing phosphatidylcholine were scraped from the plates, extracted with 3 mL of chloroform and methanol, and vortexed for 1 min; then 2.7 mL of distilled H_2O was added. The mixture was allowed to settle for 30 min and the bottom chloroform layer containing the phospholipid was removed. The quantity of phosphatidylcholine (phosphorus) was determined as described above.

Membrane fluidity. Membrane fluidity was assessed by the method of Kuhry *et al.* [22], using the apolar core probe. The probe was made as follows: 16 μL of 4.5 mM DPH was added to 4 mL of 0.05 M Tris, pH 7.4. Hepatic microsomes were diluted with either 0.05 M Tris, pH 7.4, 0.5 mM ascorbic acid, or 0.5 mM ascorbylpalmitate to 1.6 mg protein/mL and stirred in a cold room (4°) for 30 min. Samples were spun at 10,000 *g* to remove excess ascorbic acid or ascorbylpalmitate and rediluted with 0.05 M Tris, pH 7.4. Twenty microliters of the rediluted microsomes was added to 2 mL of the Tris buffer containing 8.95 μL of the probe, and incubated for 60 min at 37° in a Dubnoff shaking water bath. The fluorescence intensity was measured with an SLM 8000 spectrophotometer (excitation wavelength 340 nm; emission 450 nm) equipped to keep the cell suspension at 37°. Four polarization angles were measured: 90/90, 90/0, 0/0, and 0/90. A control of 20 μL of 1.6 mg/mL microsomal protein in 2 mL of Tris, pH 7.4, was subtracted from all polarization measurements before calculating anisotropy values.

Cholesterol content. The quantity of cholesterol in hepatic microsomes was determined by the method of Heider and Boyett [23]. Hepatic microsomes (20, 40, 60 μL of 5 mg protein/mL) were placed in 100 μL of 0.1 M K_2HPO_4 and 0.25 mL of ethanol with a final volume of 125 μL. Standards contained 0–8 μg cholesterol (CHO) in ethanol in 0.1 mL of K_2HPO_4 to a final volume of 125 μL. Ten microliters of 80 mM sodium cholate in 4% Triton X-100 was added to solubilize cholesterol in the membrane. The samples stood at room temperature for 30 min and then 1 mL of enzyme mixture (19.4 mL of 0.1 M K_2HPO_4, 0.45 mL of 0.2 units/mL cholesterol esterase, 2.0 units/mL cholesterol oxidase, 20 units/mL horseradish peroxidase and 8 mg/mL *p*-hydroxyphenylacetic acid) was added. The samples were incubated at 37° for 30 min in a shaking water bath and read on a fluorometer with an excitation of 325 nm and an emission at 415 nm. Controls contained membranes without the enzyme mixture and the enzyme mixture without the membrane.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate was performed using the discontinuous buffer system described by Laemmli [24]. Protein samples (1–10 μg) which had been treated previously with 1% SDS and 1% mercaptoethanol at 100° for 3 min were applied to a 7.5% acrylamide gel and subjected to electrophoresis in a vertical slab electrophoresis assembly (Hoefer Scientific Instruments). Electrophoresis was carried out at 50 mA/gel during separation. The gels were stained with the silver stain technique [25]. The standard proteins used as molecular weight markers were rabbit muscle phosphorylase B (92,500 daltons), bovine serum albumin (66,200 daltons), hen eggwhite

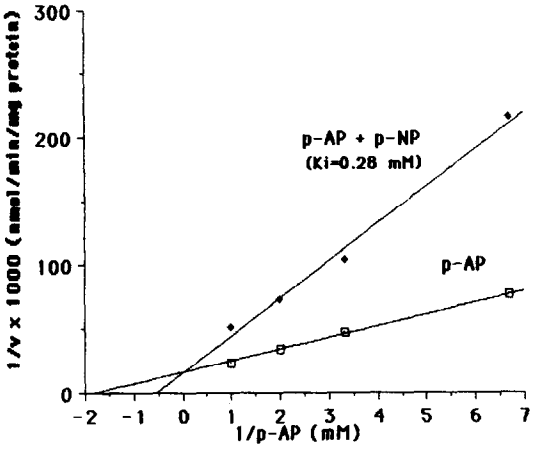

Fig. 1. Lineweaver–Burk plot of the effect of *p*-nitrophenol (p-NP) on *p*-aminophenol (p-AP) glucuronidation in hepatic microsomes isolated from ascorbate-supplemented guinea pigs. Similar kinetics were obtained in the ascorbate-deficient hepatic microsomes. *p*-Aminophenol glucuronidation was measured in the presence (◆) and the absence (□) of *p*-nitrophenol (1 mM). *p*-Aminophenol (1.0 to 0.12 mM) was added to the incubation mixture and assayed as described under Materials and Methods with modifications: 0.5 mg/mL microsomal protein was sonicated using a Branson Cell Disrupter 200 (20 kHz) for 5 min on ice at a continuous pulse and there was no Triton X-100 in the incubation mixture. N = 3 and the hepatic level of ascorbic acid in the supplemented group was 31.3 ± 5.7 mg/100 g and in the deficient 3.7 ± 1.4 mg/100 g wet liver weight.

Table 1. Ascorbic acid deficiency and hepatic UDPGT activity towards *p*-nitrophenol

Treatment	*p*-Nitrophenol (nmol/min/mg protein)	Cytochrome P-450 (nmol/100 mg protein)	Ascorbic acid (mg/100 g liver)
Deficient diet plus ascorbate	2.1 ± 0.27 (4)	84.8 ± 10.4 (4)	31.3 ± 5.7 (4)
Deficient diet	0.67 ± 0.43* (6)	36.6 ± 5.2* (6)	3.7 ± 1.4* (6)

Assay conditions for *p*-nitrophenol, cytochrome P-450 and ascorbic acid were as described under Materials and Methods. The numbers in parentheses equal the number of animals, and the data are means ± SD.
* $P < 0.001$.

ovalbumin (45,000 daltons), bovine carbonic anhydrase (31,000 daltons), soybean trypsin inhibitor (21,500 daltons), and hen eggwhite lysozyme (14,400 daltons).

Statistical methods. A two-tailed Student's *t*-test was used for all of the data analysis.

RESULTS

Hepatic microsomes from guinea pigs fed an ascorbate-deficient diet had a 68% decrease in the specific activity of UDPGT towards the substrate, *p*-nitrophenol (Table 1). Concomitant with this reduction, there was a 56% decrease in the quantity of cytochrome P-450 which had been reported earlier in ascorbic acid deficient guinea pigs [26]. The addition of ascorbic acid (0.25 to 2.0 mM) *in vitro* had no effect on UDPGT activity towards *p*-nitrophenol in either the ascorbate-supplemented or ascorbate-deficient animals.

p-Nitrophenol was found to be a competitive inhibitor of *p*-aminophenol glucuronidation with a K_i of 0.28 mM (Fig. 1). Unlike *p*-nitrophenol, in our previous study, bilirubin and acetaminophen were found to be noncompetitive and uncompetitive inhibitors of *p*-aminophenol glucuronidation respectively [13].

UDP-*N*-acetylglucosamine (UDPGLcNAC, 2.5 mM), a specific activator of UDPGT, had no significant effect on the initial rate of UDPGT in the absence or presence of Emulgen 911 in ascorbate-deficient and ascorbate-supplemented animals (Table 2). Other concentrations of UDPGLcNAC (1.0 to 5.0 mM) were also found to have no effect on UDPGT activity.

Microsomal UDPGT from ascorbate-deficient animals in the presence of Emulgen 911 was more labile to thermal inactivation than UDPGT from ascorbate-supplemented animals (Fig. 2). At 42°, microsomal UDPGT from ascorbate-deficient guinea pigs lost 41% of its activity compared to 11% in UDPGT from ascorbate-supplemented animals. Only 9% of UDPGT activity remained in the ascorbate-deficient animals at 52°, while the ascorbate-supplemented retained 31% of its original activity.

Table 2. Effect of uridine 5'-diphospho-*N*-acetylglucosamine (UDPGLcNAC) on hepatic UDP-glucuronyltransferase in the absence and presence of Emulgen 911

	UDP-glucuronyltransferase (nmol/mg protein)	
	Deficient diet plus ascorbate	Deficient diet
Without Emulgen 911		
−UDPGLcNAC		
5 min	9.0 ± 1.5 (3)	5.5 ± 1.0* (3)
20 min	15.1 ± 3.5 (3)	10.9 ± 1.8 (3)
+UDPGLcNAC (2 mM)		
5 min	10.5 ± 3.0 (4)	7.0 ± 1.5 (4)
20 min	22.4 ± 6.1 (4)	15.1 ± 2.6 (4)
With Emulgen 911		
−UDPGLcNAC		
5 min	12.0 ± 0.8 (4)	6.0 ± 0.6† (5)
20 min	18.9 ± 4.7 (4)	12.5 ± 1.3‡ (5)
+UDPGLcNAC (2 mM)		
5 min	10.8 ± 2.6 (4)	8.4 ± 2.9§ (5)
20 min	21.0 ± 5.0 (4)	17.0 ± 4.8§ (5)

The assay is described under Materials and Methods. The numbers in parentheses equal the number of animals. Data are means ± SD.
* $P < 0.05$ vs deficient diet plus ascorbate.
† $P < 0.001$ vs deficient diet plus ascorbate.
‡ $P < 0.02$ vs deficient diet plus ascorbate.
§ Not significant.

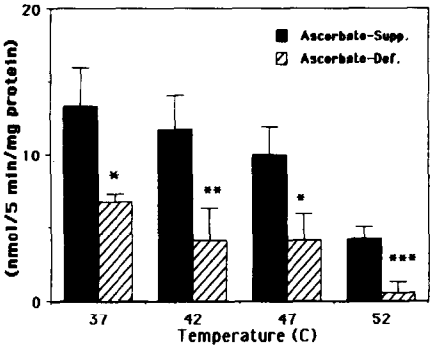

Fig. 2. Thermal inactivation of Emulgen 911 soluble UDPGT from ascorbate-supplemented and -deficient guinea pigs. Assay conditions were as described under Materials and Methods with modifications: microsomes treated with Emulgen 911 (0.67 mg detergent/mg protein) were spun for 15 min at 100,000 *g*. The supernatant fraction was incubated in a water bath at each temperature for 1 min, and then placed on ice for 1 min before being assayed for *p*-nitrophenol activity. The hepatic level of ascorbic acid in the supplemented group (■) was 31.3 ± 5.7 mg/100 g and in the deficient (▨) 3.7 ± 1.4 mg/100 g wet liver weight. Data are means ± SD, N = 4. Key: * $P < 0.01$, ** $P < 0.02$, and *** $P < 0.001$.

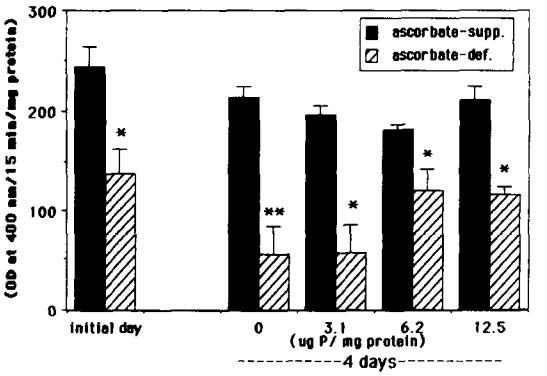

Fig. 3. Effect of phosphatidylcholine on UDPGT activity eluted from a DEAE-cellulose column. UDPGT from hepatic microsomes was partially purified, and *p*-nitrophenol activity was assayed as described in Materials and Methods. DEAE-cellulose eluant from both ascorbate-supplemented (■) and ascorbate-deficient (▨) guinea pig livers was stored on ice at 4° for 4 days and reassayed for activity towards *p*-nitrophenol in the presence and absence of phosphatidylcholine (0–25 μg P/mg protein). The data are means (±SD) of two experiments; the hepatic levels of ascorbic acid were 31 ± 8 mg/100 g in the supplemented animals and 1.6 ± 1.3 mg/100 g wet liver weight in tle deficient. Key: * $P < 0.05$, and ** $P < 0.02$.

Qualitative differences were also apparent during the purification of UDPGT. While both groups of animals lost considerable UDPGT activity during the purification procedure, UDPGT activity from ascorbate-deficient guinea pigs eluted from the high-affinity UDP-glucuronic acid agarose column chromatography was often undetectable (Table 3). The *in vitro* addition of ascorbic acid (0.25 to 2.0 mM) or phosphatidylcholine [6 to 25 μg P (phosphorus)/mg protein] could not stabilize UDPGT activity from the ascorbate-deficient animals. In addition, ascorbate-deficient guinea pig UDPGT eluted from a DEAE-cellulose column and stored for 4 days on ice at 4° lost over 50% of its activity whereas the ascorbate-supplemented lost only 10% (Fig. 3). However, when phosphatidylcholine (6.2 to 12.5 μg P/mg protein) was added to ascorbate-deficient UDPGT fractions which had lost activity upon storage, the level of activity was reconstituted but never approached that found in the ascorbate-supplemented fractions (Fig. 3). Higher concentrations of phosphatidylcholine (25–100 μg P/mg protein) had

Table 3. Purification of hepatic microsomal UDPGT from ascorbate-supplemented and ascorbate-deficient guinea pigs

Fraction	Total activity [nmol·min^{-1}·mL^{-1}]	Total protein	Specific activity [nmol·min^{-1}·(mg protein)$^{-1}$]
Ascorbic acid-supplemented microsomes	31	76	0.4
E-911 Soluble microsomal protein	80	76	1.1
DEAE-Cellulose	21	12	1.8
DEAE-Sephadex	8	3	2.7
UDP-Glucuronic acid agarose	7	0.7	10
Ascorbic acid-deficient microsomes	34	75	0.5
E-911 Soluble microsomal protein	34	75	0.5
DEAE-Cellulose	10	11	0.9
DEAE-Sephadex	4	4	1.0
UDP-Glucuronic acid agarose	0.7	0.6	1.2

The purification procedure was carried out as described under Materials and Methods. The data represent the average of 4 fractionations for each animal group.

Table 4. Microsomal membrane fluidity in the presence and absence of ascorbic acid and ascorbylpalmitate

Treatment	Anisotropy		
	AA	+AA (0.5 mM)	+AP (0.5 mM)
Deficient diet plus ascorbate	4.4 ± 0.3 (12)	4.7 ± 0.3 (4)	4.2 ± 0.3 (4)
Deficient diet	5.1 ± 0.4* (13)	5.1 ± 0.6 (4)	4.1 ± 0.4 (4)

The assay for membrane fluidity is described under Materials and Methods. The numbers in parentheses equal the number of animals and the data are means ± SD. AA = ascorbic acid; AP = ascorbylpalmitate.
* $P < 0.001$.

Table 5. Cholesterol, total phospholipid, and phosphatidylcholine content of hepatic microsomes from ascorbate-supplemented and ascorbate-deficient guinea pigs

Treatment	Cholesterol (μmol/mg protein)	Total phospholipid (μmol P/100 mg protein)	Phosphatidylcholine (μmol P/100 mg protein)
Deficient diet plus ascorbate	0.034 ± 0.007 (4)	109.3 ± 7.4 (3)	17.2 ± 5.2 (3)
Deficient diet	0.029 ± 0.003* (6)	116.1 ± 7.1* (3)	16.9 ± 4.0* (3)

Assay conditions are described under Materials and Methods. The numbers in parentheses equal the number of animals, and the data are means ± SD.
* Not significant.

no effect on UDPGT activity or were slightly inhibitory.

Microsomes from ascorbate-deficient guinea pigs had a statistically significant increase of 14% in membrane fluidity (Table 4). This could not be reversed with the *in vitro* addition of ascorbic acid. However, ascorbylpalmitate, a more lipophilic form of the vitamin, could reverse the increase in membrane fluidity found in microsomes from ascorbate-deficient animals. Palmitic acid had no effect on membrane fluidity. Furthermore, there were no differences in the quantity of cholesterol, total phospholipid, or phosphatidylcholine of hepatic microsomes from the ascorbate-supplemented and ascorbate-deficient guinea pigs (Table 5).

UDPGT partially purified from ascorbate-deficient and ascorbate-supplemented guinea pigs was subjected to SDS–PAGE to determine if quantitative differences existed in the respective enzymes. There was a consistent decrease in banding in the 55,000 dalton region with the ascorbic acid deficient guinea pig UDPGT compared to the ascorbate-supplemented (Fig. 4). Previous studies indicated that the guinea pig UDPGT isozyme with activity towards *p*-nitrophenol had a molecular weight of 55,000 daltons when subjected to SDS–PAGE [4]. Furthermore, there was no detectable flavin-containing monooxygenase activity towards dimethylaniline or cytochrome P-450 as determined by the reduced carbon monoxide binding spectrum in UDPGT fractions eluted from the high-affinity UDP-glucuronic acid agarose column.

DISCUSSION

Previous studies showed a reduction in specific activity towards *p*-aminophenol whereas bilirubin and acetaminophen glucuronidation were unaffected, suggesting that one isozyme of UDPGT is altered in ascorbate deficiency [13]. In the present study, the glucuronidation of another planar phenol, *p*-nitrophenol, was also jeopardized in ascorbate deficiency. Burchell [27] found that activities towards 2-aminophenol and 4-nitrophenol copurified, maintaining approximately the same ratio of specific activities over five purification steps. In keeping with this, the present study found *p*-nitrophenol to be a competitive inhibitor of *p*-aminophenol glucuronidation. In contrast, previous studies showed that acetaminophen and bilirubin were uncompetitive and noncompetitive inhibitors of *p*-aminophenol glucuronidation respectively [13]. The selective effect of the vitamin on hepatic glucuronidation is in keeping with the findings that only certain isozymes of cytochrome P-450 are jeopardized in ascorbate deficiency [26].

As a possible mechanism to explain the reduction in UDPGT activity in ascorbate deficiency, the effect of UDP-*N*-acetylglucosamine (UDPGLcNAC) was examined. UDPGLcNAC is a specific activator of UDPGT which does not perturb the membrane and may allosterically affect the UDP-glucuronyltransferase [28] or assist the transport of the cofactor, UDPGA, through an impermeable phospholipid barrier [29]. In previous studies, UDPGLcNAC activated UDP-glucuronyltransferase in the guinea pig towards many substrates including *p*-nitrophenol [30]. In contrast, the present study indicated that UDPGLcNAC had no effect on the initial rate of UDPGT in either the ascorbate-supplemented or ascorbate-deficient animals (Table 2). A possible explanation is that UDPGLcNAC, like other sulfhydryl agents, may maintain a linear enzymatic rate.

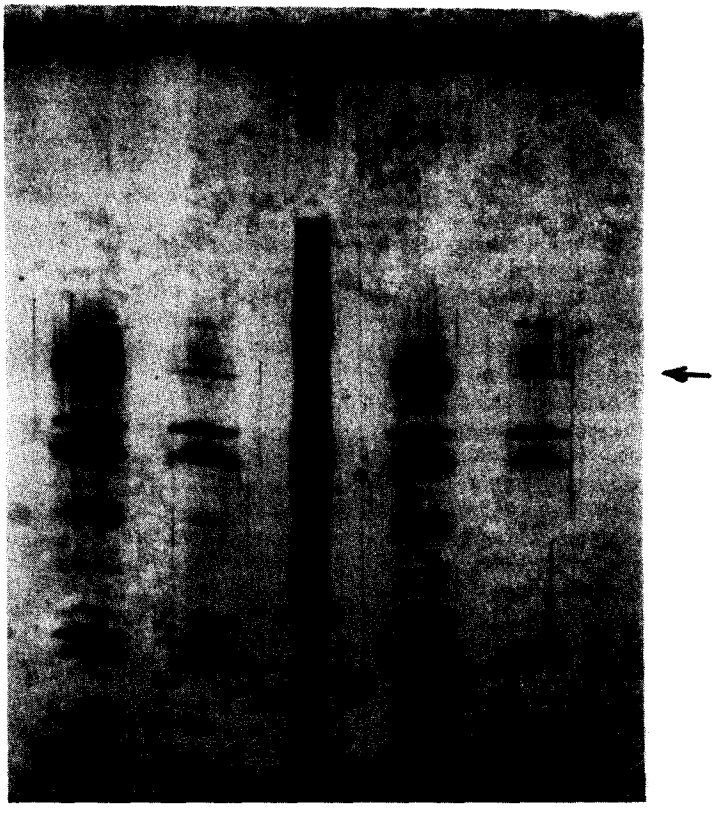

Fig. 4. SDS-electrophoresis of partially purified hepatic UDPGT from ascorbate-supplemented and -deficient guinea pigs (N = 4). Electrophoresis was performed in 7.5% polyacrylamide gel as described in Materials and Methods. The direction of migration was from top to bottom. Protein was loaded as follows: (left to right) lane 1, ascorbic acid supplemented, 8 μg; lane 2, ascorbic acid deficient, 8 μg; lane 3, protein standards, 5 μg; lane 4, ascorbate-supplemented, 6 μg; and lane 5 ascorbate-deficient, 6 μg. The hepatic level of ascorbic acid in the supplemented group was 31.3 ± 5.7 mg/100 g while the deficient was 3.7 ± 1.4 mg/100 g wet liver weight. The standard proteins used as molecular weight markers were rabbit muscle phosphorylase B (92,500 daltons); bovine serum albumin (66,200 daltons); hen eggwhite ovalbumin (45,000 daltons); bovine carbonic anhydrase (31,000 daltons); soybean trypsin inhibitor (21,500 daltons); and hen eggwhite lysozyme (14,400 daltons).

Past studies often assayed UDPGT activity at 30 or 60 min, and the reported activation by UDPGLcNAC may be due to prolongation of the enzymatic rate of the reaction [28–30].

Evidence has been provided indicating that both qualitative and quantitative alterations occur in microsomal and partially purified UDPGT from ascorbate-deficient guinea pigs. Qualitative changes found in UDPGT from ascorbate-deficient animals included an increased lability to thermal inactivation, storage of DEAE-cellulose fractions at 4°, purification with UDP-glucuronic acid agarose column chromatography, and increased microsomal membrane fluidity. Qualitative differences have also been reported in other enzymes jeopardized in ascorbate deficiency. The flavin-containing monooxygenase partially purified from ascorbate-deficient guinea pigs is more dependent on the addition of exogenous FAD for optimal activity, has enhanced susceptibility to substrate inhibition, and is more labile upon freezing [19]. In addition, cytochrome P-450, another microsomal enzyme jeopardized in deficiency, had alterations in both type I and type II substrate binding spectra in ascorbate-deficient guinea pigs [31, 32]. It should be noted, however, that qualitative alterations found in ascorbate-deficient animals are relatively specific since other microsomal electron transport components such as cytochrome b_5 and cytochrome b_5 reductase are not affected [33].

These qualitative alterations may be due to subtle conformational changes in the secondary or tertiary structure of UDPGT in ascorbate-deficient animals resulting in a catalytically impaired enzyme. One possible explanation is that the increase in membrane fluidity in ascorbate deficiency may change important hydrophobic interactions between membrane phospholipids and UDPGT, resulting in a structurally unstable enzyme or enzyme complex. Both excessive increases or decreases in membrane fluidity have been shown to be deleterious to UDPGT activity [34]. UDPGT may be sensitive to changes in protein–lipid interactions because it is an oligomer whose activity is

dependent on the proper association of monomeric polypeptides [2, 3]. Thus, alterations in the membrane environment may result in the disaggregation of these important components of UDPGT resulting in a reduction in catalytic activity. The use of a more specific fluorescent probe which binds directly to UDPGT would help determine whether phospholipid membrane environment immediately surrounding UDPGT is also altered. Furthermore, the fact that ascorbylpalmitate could restore membrane fluidity in the ascorbate-deficient animals suggests a role for the vitamin in maintaining the integrity of the phospholipid environment. A possible explanation is that the lipophilic form of the vitamin is an effective carrier of ascorbic acid across the lipoid-microsomal membrane, thus allowing the vitamin to associate with membrane phospholipids. These findings are in keeping with previous studies which showed that only the lipophilic form of ascorbic acid could reverse alterations found in the type II aniline-cytochrome P-450 binding spectra in ascorbic acid deficient guinea pigs [32].

In addition to these qualitative differences, there was a quantitative loss of UDPGT protein at 55,000 daltons in ascorbate deficiency when partially purified fractions subjected to SDS–PAGE were compared to the ascorbate-supplemented. In keeping with this, both the flavin-containing monooxygenase [19] and cytochrome P-450 [26] were found to be quantitatively reduced in ascorbate-deficient animals. This quantitative loss of the cytochrome P-450 [26] and the flavin-containing monooxygenase [19] of the protein was not due to an alteration in protein synthesis, since ascorbate-deficient guinea pigs were shown to have the same fold induction with phenobarbitol treatment. However, since UDP-glucuronyltransferase is regulated by a different operon than cytochrome P-450 [35], a reduction in protein synthesis cannot be ruled out at the present time. It seems unlikely that excessive oxidation of membrane lipids is responsible for the quantitative loss of UDPGT protein since lipid peroxidation was found to be 30% higher in the ascorbate-supplemented animals compared to the ascorbate-deficient animals [36]. In addition, enhanced release of degradative enzymes from lysosomes also seems unlikely to explain this quantitative loss in UDPGT protein since this degradative effect would be non-specific resulting in a quantitative loss of all proteins in the microsomal membrane. In ascorbate deficiency, there is a selective loss of certain isozymes of specific proteins like cytochrome P-450 [26].

The role of dietary ascorbic acid is complex and multifactorial in that it protects against both qualitative alterations in UDPGT structure and a quantitative loss of the enzyme protein. A cascade of deleterious alterations may occur in ascorbate-deficient animals in which qualitative or conformational alterations in UDPGT structure make the enzyme more vulnerable to inactivation and degradation. Because UDPGT is quantitatively the most important detoxifying enzyme in mammalian metabolism, understanding factors such as dietary ascorbate which influence its activity is important because decreases could result in enhanced toxicity of environmental chemicals, pharmaceutical agents, or endogenous compounds.

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