Studies on drug-induced lipidosis: subcellular localization of phospholipid and cholesterol in the liver of rats treated with chloroquine or 4,4′-bis (diethylaminoethoxy)α,β-diethyldiphenylethane[1]

Yuji Matsuzawa and Karl Y. Hostetler[2]

Department of Medicine, Veterans Administration Medical Center and the University of California, San Diego, CA 92161

Abstract Administration of chloroquine or 4,4′-bis(diethylaminoethoxy)α,β-diethyldiphenylethane (DH) to rats in oral doses of 100 mg/kg for 7 days causes phospholipid and cholesteryl ester accumulation in liver. To further characterize this drug-induced lipidosis, we have isolated and characterized the lipids of subcellular fractions from control rats and rats treated with chloroquine, DH, and Triton WR-1339. The phospholipid content of liver is increased 1.5-fold by chloroquine or DH treatment but is unaffected by Triton WR-1339. Acid phosphatase is increased by treatment with these three agents. Chloroquine and DH cause a shift of acid phosphatase from the light mitochondrial fraction (L) to the heavy mitochondrial fraction (M). Multilamellar bodies, an ultrastructural hallmark of chloroquine and DH-induced lipidosis, were isolated in a highly-purified form from the M fraction of chloroquine- or DH-treated rats. They are highly enriched in acid phosphatase indicating their lysosomal origin. In addition, they contain large amounts of phospholipid, cholesterol, and cholesteryl ester and are the sole site of bis(monoacylglycero)phosphate and the enzyme which catalyzes its synthesis from phosphatidylglycerol. Analysis of the phospholipid content of the respective control and drug-treated liver fractions shows that the entire excess phospholipid content of chloroquine- or DH-treated liver can be accounted for by the drug-induced multilamellar bodies. Triton WR-1339-induced lysosomes, which were isolated for comparison, also contain bis(monoacylglycero)phosphate and bis(monoacylglycero)phosphate synthetase. However, they differ from the drug-induced lysosomes in that their sphingomyelin content is much higher and their total phospholipid and phosphatidylinositol content much lower. The multilamellar bodies are the principal intracellular site of accumulation of chloroquine and DH, respectively. Increased delivery of phospholipid to lysosomes and decreased lysosomal catabolism of phospholipid are the factors which are thought to cause this experimental lipidosis. High levels of phosphatidylinositol in the multilamellar body may be in part responsible for the increased content of bis(monoacylglycero)phosphate since it has been identified as an acyl donor in bis(monoacylglycero)phosphate synthesis.—**Matsuzawa, Y., and K. Y. Hostetler.** Studies on drug-induced lipidosis: subcellular localization of phospholipid and cholesterol in the liver of rats treated with chloroquine or 4,4′-bis(diethylaminoethoxy)α,β-diethyldiphenylethane. *J. Lipid Res.* 1980. **21:** 202–214.

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The administration of certain cationic amphiphilic drugs can cause phospholipid storage in body tissues. In one of the first examples of this form of drug toxicity, the chronic administration of 4,4′-bis(β-diethylaminoethoxy)α,β-diethyldiphenylethane (DH) to patients was found to cause a phospholipidosis characterized by the accumulation of intracellular multilamellar bodies and by the appearance in the bone marrow of foamy histiocytes which resemble those found in Niemann-Pick disease (1, 2). The tissue concentration of all phospholipids was increased in liver, spleen and other tissues, but the tissue content of bis(monoacylglycero)phosphate (also known as lysobisphosphatidic acid) was increased many fold more than other phosphoglycerides (1, 2). The concentration of acidic phospholipids in liver correlated with the degree of accumulation of the drug and its metabolites (3). Administration of DH to rats or monkeys caused an increase in the tissue content of phospholipids and bis(monoacylglycero)phosphate and was accompanied by the formation of multilamellar bodies in the cytoplasm (4). Chloroquine administration to rats also causes a substantial increase in liver phospholipid content and bis(monoacylglycero)phosphate accumulation is particularly marked (5). It is

Abbreviations: DH, 4,4′-bis(diethylaminoethyoxy)α,β-diethyldiphenylethane.

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[2] To whom correspondence should be addressed.

known to inhibit the degradation of ^{125}I-labeled low density lipoprotein (LDL) in cultured skin fibroblasts but has no effect on the binding of LDL to the cell surface receptors (6). Chloroquine also inhibits the in vitro degradation of radiolabeled LDL and VLDL by a liver postnuclear supernatant at pH 4.4 (7), and also inhibits various other lysosomal acid hydrolases presumably by raising the intralysosomal pH (8, 9). However, the metabolic basis for the marked accumulation of bis(monoacylglycero)phosphate and other phospholipids in the liver of chloroquine-treated rats is unknown.

In liver, bis(monoacylglycero)phosphate is found only in lysosomes as first demonstrated by Wherret and Huterer (10). The studies of Renkonen and coworkers with BHK-21 cells (11, 12) and of Mason, Stossel, and Vaughan (13) with alveolar macrophages also indicate a lysosomal localization of bis(monoacylglycero)phosphate. Electron microscopic study of liver tissue from humans (14, 15) or rats (16, 17) treated with DH or chloroquine indicates that a prominent feature of the lipidosis is the presence in the cytoplasm of numerous autophagic vacuoles and multilamellar bodies (also called myelin figures). These multilamellar bodies are of lysosomal origin based on the histochemical demonstration of acid phosphatase activity (15–17) and they are thought to be rich in phospholipid, based on their histochemical properties (14, 16, 17).

Numerous other drugs with widely differing pharmacologic effects also cause the accumulation of phospholipids in body tissues. The other agents include chlorophenteramine, fenfluramine, triparanol, trans-1,4-bis(2-chlorobenzylaminomethyl)cyclohexane, azacosterol, 1-chloroamitryptyline, iprindol, and chlorcyclizine (18). The drugs which cause systemic phospholipidosis have certain structural features in common. An uncharged hydrophobic region is present (usually an aromatic ring structure) and an amine group is found at varying distances from the hydrophobic region. It is believed that the ability of these drugs to cause phospholipidosis is related to their structural similarities rather than to any common pharmacologic action (19, 20).

Lysosomes isolated from the liver of rats treated with chloroquine or DH have been shown to be enriched in bis(monoacylglycero)phosphate (21, 22). In these studies, no increase in liver total phospholipid was found with DH while a slight increase (10%) was noted with chloroquine; total cholesterol was not increased with chloroquine treatment but no data are presented for DH (21). The intracellular sites of phospholipid accumulation in chloroquine-treated liver cannot be determined in these studies since the other membrane pools of phospholipid were not analyzed; marker enzyme determinations were not presented to indicate the degree of purity of the lysosomal fractions (21, 22).

In this publication, we report studies of the subcellular distribution of phospholipids and cholesterol in normal rat liver and in the liver of rats treated with Triton WR-1339, chloroquine, or DH. Mitochondria, microsomes, and highly-purified multilamellar bodies (lysosomes) have been isolated and the purity of these fractions has been assessed by electron microscopic analysis and by marker enzyme determinations. The lipid composition of the normal subcellular fractions has been compared with that of the rats treated with Triton WR-1339, chloroquine, and DH. Our results indicate that the increased hepatic content of phospholipid caused by chloroquine and DH can be accounted for by the presence of these multilamellar lysosomes which also contain the respective drugs in high concentrations. These lysosomes contain large amounts of bis(monoacylglycero)-phosphate and the enzyme required for its synthesis.

METHODS

Preparation of subcellular fractions

Male Sprague-Dawley rats were given chloroquine phosphate (Aralen®) or 4,4′-bis(diethylaminoethoxy)-α,β-diethyldiphenylethane (DH) for seven days by stomach tube (100 mg/kg). Triton WR-1339 was dissolved in normal saline and a single dose of 850 mg/kg was injected intravenously into male rats 4 days prior to sacrifice. After an overnight fast, the rats were killed and the livers were removed, minced, and washed with cold 0.25 M sucrose containing 5 mM tris(hydroxymethyl)aminomethane-HCl (pH 7.4) and 2 mM EDTA. The following operations were carried out at 4°C. A 10% (w/v) homogenate was prepared in 0.25 M sucrose/5 mM Tris/2 mM EDTA by 3 to 4 strokes with a motor-driven Potter-Elvehjem homogenizer with a Teflon pestle. Subcellular fractions from the control or Triton WR-1339 treated liver homogenates were isolated as described by Sarzala et al. (20). Subcellular fractions from chloroquine or DH-treated rats were isolated by the following modifications. After nuclei and cell debris (N) had been pelleted by centrifugation at 800 *g* for 5 min, the postnuclear supernatant was separated into a heavy mitochondria pellet (M) and a postmitochondrial supernatant by centrifugation at 9,000 *g* for 10 min. Pure mitochondria and lysosomes were isolated from the M pellet by the following procedure. The M fraction was resuspended in 0.25 M sucrose/5 mM Tris-HCl (pH

7.4) and 5 ml was layered on the top of a 28 ml continuous sucrose gradient (0.61–1.60 M) and a 5 ml cushion of 1.75 M sucrose and centrifuged at 82,000 *g* for 30 min in a SW-27 rotor (Beckmann Instruments). With chloroquine-treated liver, two upper bands, designated as lysosome I and II, and a heavy lower band consisting of mitochondria were obtained. In the rats treated with DH, a single lysosomal band and a single mitochondrial band were obtained. Lysosomes were purified from the M + L fraction of Triton WR-1339-treated rat liver by selective flotation as previously described by Trouet (23).

The respective bands representing lysosomes or mitochondria were harvested with a curved needle, diluted carefully to a concentration of 0.25 M sucrose with cold water with constant stirring and sedimented in a Sorvall SS-34 rotor by centrifugation at 34,000 *g* for 15 min. The pellets were resuspended in 0.25 M sucrose/5 mM Tris-HCl. Light mitochondria (L) were sedimented by centrifugation of the postmitochondrial supernatants at 20,000 *g* for 10 min. Microsomes (P) were isolated from the resulting supernatant by centrifugation at 110,000 *g* for 60 min in a 60 Ti rotor (Beckman Instruments). The supernatant was decanted and reserved. All fractions were stored frozen at −60°C until use. The following marker enzymes were determined in the respective subcellular fractions: succinate dehydrogenase (mitochondria) was determined by the method of Green, Mii, and Kohout (24); β-glycerol phosphatase (lysosomes) as described by Trouet (23) and rotenone-insensitive NADPH-cytochrome C reductase (microsomes) by the method of Sottacasa et al. (25). Synthesis of bis(monoacylglycero)P was assayed as described previously (26): the incubation mixture contained 50 mM sodium acetate buffer (pH 4.4), 10 mM β-mercaptoethanol, 4×10^{-6} M phosphatidyl[1′,3′-^{14}C]glycerol, specific activity, 128 mCi/mmol, and 100 to 200 mg of the respective protein fractions in a total volume of 0.200 ml. The incubations were carried out for 1 hr at 37°C. The reactions were stopped by the addition of 20 volumes of chloroform–methanol 2:1 (by volume) and the lipids were extracted and washed by the method of Folch, Lees, and Sloane Stanley, (27) and analyzed by thin-layer chromatography on 0.25 mm plates of silica gel G prepared with 0.4 M boric acid and developed in chloroform–methanol–concentrated ammonia–water 70:30:3:2 (by volume) as previously described (26).

Electron microscopy

The membrane fractions were prepared for electron microscopic examination as described by Cotman and Flansburg (28). Briefly, 50 μg of protein was fixed with buffered 1% osmium tetroxide in a pencil-neck, flat bottom capsule. After 16 hr at 4°C, the capsules were placed in adapters and the membranes were sedimented by centrifugation in a Beckmann SW 50.1 rotor at 15,000 *g* for 20 min. The pellets were washed three times with cold buffer and dehydrated with a graded series of ethanol water mixtures. The dehydrated pellets were carefully removed from the tip of the capsules and embedded in flat molds with Epon 812. After curing, ultrathin sections were prepared with a Sorvall MT-2 ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Zeiss EM-10 electron microscope.

Other analytical methods

The total lipid extracts of homogenates and the respective subcellular fractions were prepared and washed with 0.73% NaCl as described by Folch et al. (27). Phospholipid composition was determined by thin-layer chromatography as described by Poorthuis, Yazaki, and Hostetler (29). The total lipids (approximately 500 nmoles of lipid phosphorus) were applied to silica gel G plates impregnated with 0.4 M boric acid and developed in the first dimension with chloroform–methanol–water–concentrated ammonia 70:30:3:2 (by volume) and in the second dimension with chloroform–methanol–water 65:35:5 (by volume) (29). After visualization of the lipids with iodine vapors, the silica areas were scraped into tubes and lipid phosphorus was determined by the method of Rouser, Fleischer, and Yamamoto (30). Cholesterol and desmosterol were analyzed by gas–liquid chromatography as described by Ishikawa et al. (31). After the addition of the coprostanol internal standard to total lipid extracts representing 1–5 mg protein of the respective subcellular fractions, the extracts were evaporated to dryness with a nitrogen stream and the residue was dissolved in a small amount of hexane. The samples were analyzed with a Varian 3700 series gas chromatograph using a 100 cm column of 3% SP-2250 on 80–100 mesh Supelcoport. The column temperature was 230°C; nitrogen was used as carrier gas at a flow rate of 30 ml/min. Cholesterol was determined by an internal standard method using a Varian CDS-111 automatic data system. For total cholesterol measurement, esterified cholesterol was hydrolyzed with tetramethylammonium hydroxide prior to gas–liquid chromatography (31). Free cholesterol was measured on a aliquot which had not been subjected to hydrolysis and the esterified cholesterol content was determined by difference. DH and its metabolites in the total lipid extract were also analyzed by gas–liquid chromatography using the same column

as used for the cholesterol determinations. However, for the analysis of DH and its derivatives, a column temperature of 240° was used; otherwise, the conditions were identical to those described above. The retention times of DH, hydroxylated DH, cholesterol and desmosterol relative to that of coprostanol were 2.05, 5.67, 1.17, and 1.42, respectively. Chloroquine was determined by fluorescence using an Amicon spectrophotofluorimeter (285 nm, excitation and 397 nm, emission).

Chemicals

Triton WR-1339 was obtained from Supelco, Bellefonte, PA. Chloroquine phosphate (Aralen®) was the gift of the Sterling-Winthrop Research Institute, Rensselaer, NY. 4,4′-bis(β-diethylaminoethoxy)α,β-diethyldiphenylethane was the gift of the Torii Pharmaceutical Co., Tokyo. Silica gel G was obtained from EM Reagents, Elmsford, NY. Other chemicals were of analytical reagent grade from usual commercial sources; chloroform and methanol were redistilled before use.

RESULTS

In order to further characterize the hepatic phospholipidosis induced by chloroquine and DH, quantitative differential centrifugation of the respective liver homogenates was carried out as described in the Methods, and the following fractions were isolated and characterized; nuclei (N), heavy mitochondria (M), light mitochondria (L), microsomes (P) and supernatant (S). The subcellular distribution of various marker enzymes and total phospholipid was determined in these fractions.

Subcellular localization of marker enzymes

Fig. 1 shows the activity of the respective marker enzymes plotted as relative specific activity versus percent protein as suggested by de Duve et al. (32). Data from untreated control rats and Triton WR-1339-treated rats have been included for comparison. The highest relative specific activity and total activity of succinate dehydrogenase was found in the mitochondrial (M) fraction, and rotenone-insensitive NADPH-cytochrome C reductase activity was highest in the microsomal fraction (Fig. 1). The profile of these two marker enzymes was not substantially different in the fractions from control, Triton WR-1339-, chloroquine-, and DH-treated rats. Recovery of succinate dehydrogenase and NADPH-cytochrome C reductase was satisfactory, ranging from 82–97%, respectively.

In contrast, the distribution of acid phosphatase activity was substantially affected by treatment with chloroquine or DH. In control and Triton WR-1339-treated liver, the relative specific activity of acid phosphatase was highest in the light mitochondrial fraction (L) in agreement with previous reports (26, 32). However, in chloroquine- or DH-treated livers, the heavy mitochondrial fraction (M) had the highest relative specific activity of acid phosphatase (Fig. 1). Triton WR-1339, chloroquine, and DH treatment caused an increase in the total activity of acid phosphatase in the homogenate. The recovery of acid phosphatase activity ranged from 100 to 110%.

Bis(monoacylglycero)phosphate synthetase activity in subcellular fractions

We determined the subcellular localization of bis(monoacylglycero)phosphate synthesis by incubating [^{14}C]phosphatidylglycerol with the respective subcellular fractions at pH 4.4; the conversion of [^{14}C]-phosphatidylglycerol to [^{14}C]bis(monoacylglycero)-phosphate was determined as previously described (26). The distribution of bis(monoacylglycero)phosphate synthetase in each case was essentially identical to that of acid phosphatase. With drug treatment, this enzyme activity was shifted from the light mitochondrial fraction (L) to the heavy mitochondrial fraction (M) and the shift was slightly more pronounced with chloroquine treatment than with DH treatment. The activity of bis(monoacylglycero)phosphate synthetase in the homogenate was not affected by Triton WR-1339 treatment (Fig. 1). However, treatment with chloroquine or DH caused a 6-fold and 4-fold increase, respectively, in the total activity of this enzyme in the homogenate. Most of this activity was present in the mitochondrial (M) fraction. The recovery of bis(monoacylglycero)P synthetase activity was substantially greater than 100% in all cases. This is due in all likelihood to competition for the [^{14}C]phosphatidylglycerol substrate by the various membrane components of the homogenate resulting in a lowered apparent activity.

Subcellular distribution of phospholipids

As shown in Fig. 1, treatment of rats for 7 days with 100 mg/kg of chloroquine or DH results in a 50% increase in total liver phospholipid content, while treatment with Triton WR-1339 has no effect on total phospholipid. In normal liver, the microsomal (P) fraction accounts for about 50% of the total phospholipid. Treatment with chloroquine or DH results in substantial increases in the phospholipid content of the mitochondrial (M) fraction (twofold or greater) while little effect is noted on the total phospholipid

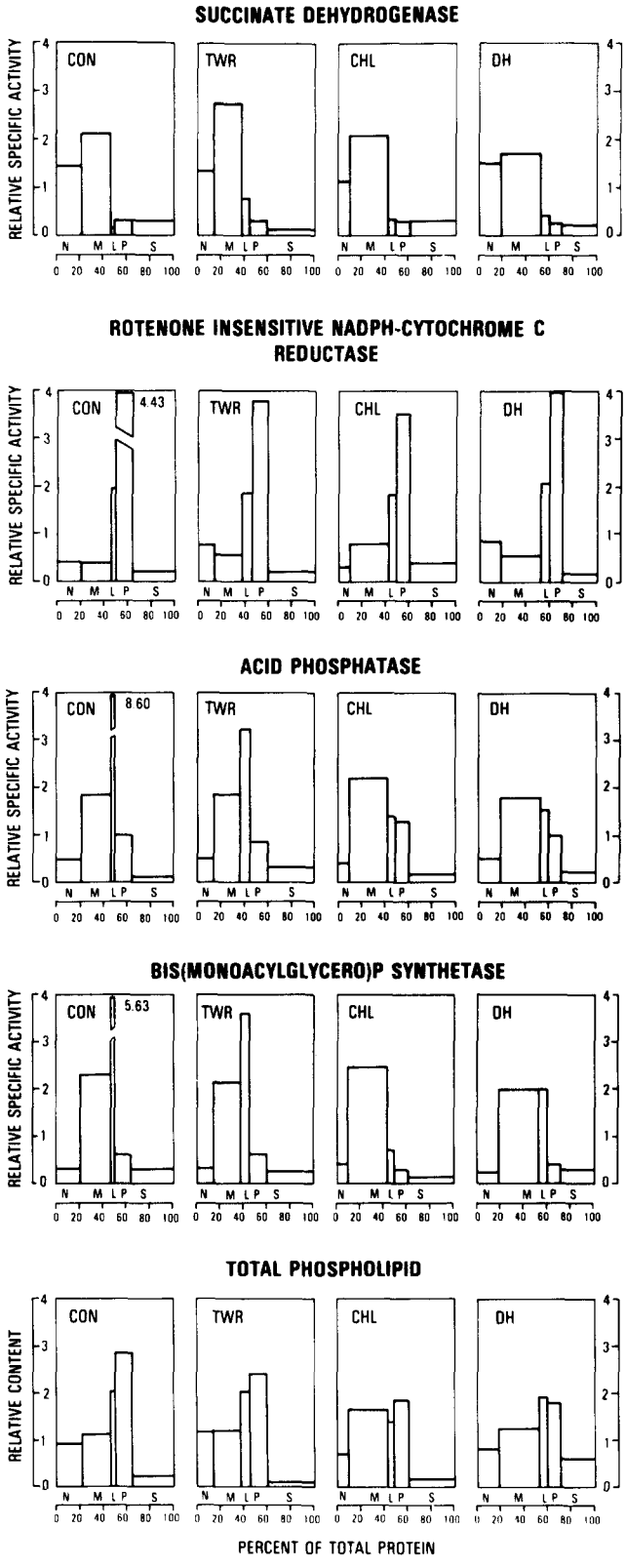

Fig. 1. Distribution of marker enzymes bis(monoacylglycero)-phosphate synthetase and total phospholipid content in rat liver subcellular fractions. Each fraction is represented on the ordinate scale by its relative specific activity (percentage of total recovered activity/percentage of total protein) or its relative content (percentage of total recovered lipid phosphorus/percentage of total protein). On the abcissa scale, each fraction is represented (cumulatively from left to right) by its protein content expressed as percentage of total recovered protein. Homogenate values: Succinate dehydrogenase (μmol min^{-1} g^{-1}) C 7.98, TWR 5.24, CHL 6.02, DH 7.08; Rotenone-insensitive NADPH cytochrome C reductase (μmol min^{-1} g^{-1}) C 19.10, TWR 15.95, CHL 17.30, DH 12.04; acid phosphatase (μmol min^{-1} g^{-1}) C 28.2, TWR 46.0, CHL 59.0; DH 49.3; BMP synthetase, (nmol min^{-1} g^{-1}) C 0.38, TWR 0.33, CHL 2.27, DH 1.60. Phospholipid data are given in Table 3. Recoveries: protein, 87–107%; succinate dehydrogenase, 82–97%; NADPH cytochrome C reductase, 79–97%; acid phosphatase, 100–110%; BMP synthetase, 108–121% and phospholipid, 85–114%. For simplicity, the data shown in Fig. 1 and in Tables 1–3 represent the results from single experiments, representative of three or more separate subcellular fractionations of the four respective rat liver treatments that were used, except that cholesterol and cholesterol ester concentrations were determined in only two of the experiments, one of which is shown. Abbreviations: C, control; TWR, Triton WR-1339; CHL, chloroquine; DH, 4,4′-bis(β-diethylaminoethoxy)α,β-diethyldiphenylethane.

content of microsomal (P) fraction or the nuclear (N) fraction. A 3- to 4-fold increase in the content of phospholipid was also noted in the supernatant of the chloroquine- or DH-treated rat liver homogenate.

Intracellular localization of chloroquine and DH

The distribution of chloroquine and DH was also determined in the subcellular fractions of the liver of rats treated with these agents. The distribution of the respective drugs is similar to that of acid phosphatase and bis(monoacylglycero)phosphate synthetase shown in Fig. 1, suggesting that the drugs may be associated with lysosomes (data not shown).

Isolation of multilamellar bodies from chloroquine and DH-treated rat liver

The results described above indicate that the localization of the lysosomal marker enzymes in chloroquine or DH-treated rat liver is shifted from the light mitochondrial fraction (L) to the heavy mitochondrial fraction (M) and that the total phospholipid content of the mitochondrial fraction (M) is greatly increased. In addition, most of the chloroquine or DH is recovered in the M fraction. These data strongly suggest that the multilamellar bodies which are a prominent ultrastructural feature of this condition (14–17) are concentrated in the mitochondrial (M) fraction. When the heavy mitochondrial fraction from chloroquine-treated rats was applied to a continuous sucrose gradient and centrifuged as described in Methods, three major bands were observed. Two fractions (lysosomes) remained in the upper portion of the gradient (equilibrium densities, lysosomes I—1.0805 g/ml, lysosomes II—1.1080 g/ml) and the third fraction (mitochondria) was recovered near the bottom of the gradient (d 1.1889 g/ml). These fractions accounted for 15, 12, and 73% of the recovered protein, respec-

tively. With DH-treated liver, only one lysosomal fraction was obtained (d 1.0691 g/ml) accounting for 15% of recovered protein while the mitochondrial fraction (d 1.1943 g/ml) accounted for 85% of recovered protein. Purified secondary lysosomes were obtained for comparison from Triton WR-1339-treated rats as previously described by Trouet (23).

Marker enzymes and bis(monoacylglycero)phosphate synthetase were measured in the respective purified fractions and the results are shown in **Table 1.** Ten or 11% mitochondrial contamination was found in lysosomes from Triton WR-1339- or DH-treated rat liver, while the mitochondrial contamination of lysosomes I and II isolated from chloroquine-treated rat liver was 1.6% and nil, respectively. Microsomal contamination of Triton WR-1339- or DH-induced lysosomes was 4 and 7%, respectively. However, it is possible that the presence of these marker enzymes in the lysosomes reflects prior autophagy of mitochondrial or microsomal membranes rather than contamination as such. Degenerating mitochondria have been observed within autophagic vacuoles in mammalian liver following treatment with chloroquine (16) or DH (14) and cytochromes from mitochondria and microsomes have been found in Triton WR-1339 lysosomes (33).

The further purification of the crude heavy mitochondrial fraction (M) with sucrose gradient centrifugation results in a marked decrease in the acid phosphatase activity of purified mitochondria, and the specific activity of acid phosphatase in tritosomes, chloroquine lysosomes I and II, and DH lysosomes is greatly enriched over that of the corresponding homogenate, 25-fold, 27-fold, 12-fold, and 16-fold, respectively. Similarly, bis(monoacylglycero)P synthetase activity recovered in the respective lysosomal fractions is enriched 144-fold (Triton lysosomes), 23-fold (chloroquine lysosome I), 12-fold (chloroquine lysosome II) and 15-fold (DH lysosome) over the activity of the respective homogenates. The purified mitochondria and the microsomes had bis(monoacylglycero)P synthetase activities less than that of the homogenate; the activity observed in these fractions can be accounted for by contamination with lysosomes based on the activity of acid phosphatase.

Electron microscopic examination of the purified lysosomal fractions

Purified lysosomal fractions from the liver of rats treated with chloroquine or DH were examined by electron microscopy (**Fig. 2**).

Treatment with chloroquine resulted in two lysosomal fractions, designated I and II. Chloroquine lysosomes I, shown in the upper panel, consisted of

TABLE 1. Specific activity of marker enzymes in subcellular fractions

	Succinate Dehydrogenase Specific Activity[a]				Rotenone-insensitive NADPH Cytochrome C Reductase Specific Activity[a]				Acid Phosphatase Specific Activity[a]				Bis(monoacylglycero)P Synthetase Specific Activity[b]			
	C	TWR	CHL	DH	C	TWR	CHL	DH	C	TWR	CHL	DH	C	TWR	CHL	DH
Homogenate (H)	7.98	5.24	6.02	7.08	19.1	16.0	17.3	12.0	28.2	46.0	59.0	49.3	0.38	0.33	2.27	1.60
Heavy mitochondria (M)	15.4	13.2	12.7	13.3	8.12	8.21	12.4	10.5	54.4	88.3	155	132	1.02	1.35	6.95	4.93
Pure mitochondria	21.0	21.7	13.2	20.1	0.98	1.45	3.51	3.23	45.5	13.2	32.0	13.0	0.33	0.10	1.42	0.20
Lysosomes I		2.33	0.21	2.18		1.91	7.57	3.23	250	1140	1580	808		61.0	51.8	23.7
Lysosomes II			0				13.2				727				29.7	
Microsomes (P)	2.39	1.62	1.55	1.77	77.1	53.4	54.6	48.4	30.0	41.2	54.5	60.1	0.27	0.28	0.85	0.80

[a] nmol per mg of protein min^{-1}.
[b] pmol per mg of protein min^{-1}.
Abbreviations: C, control; TWR, Triton WR-1339-treated; CHL, chloroquine-treated; DH, DH-treated.

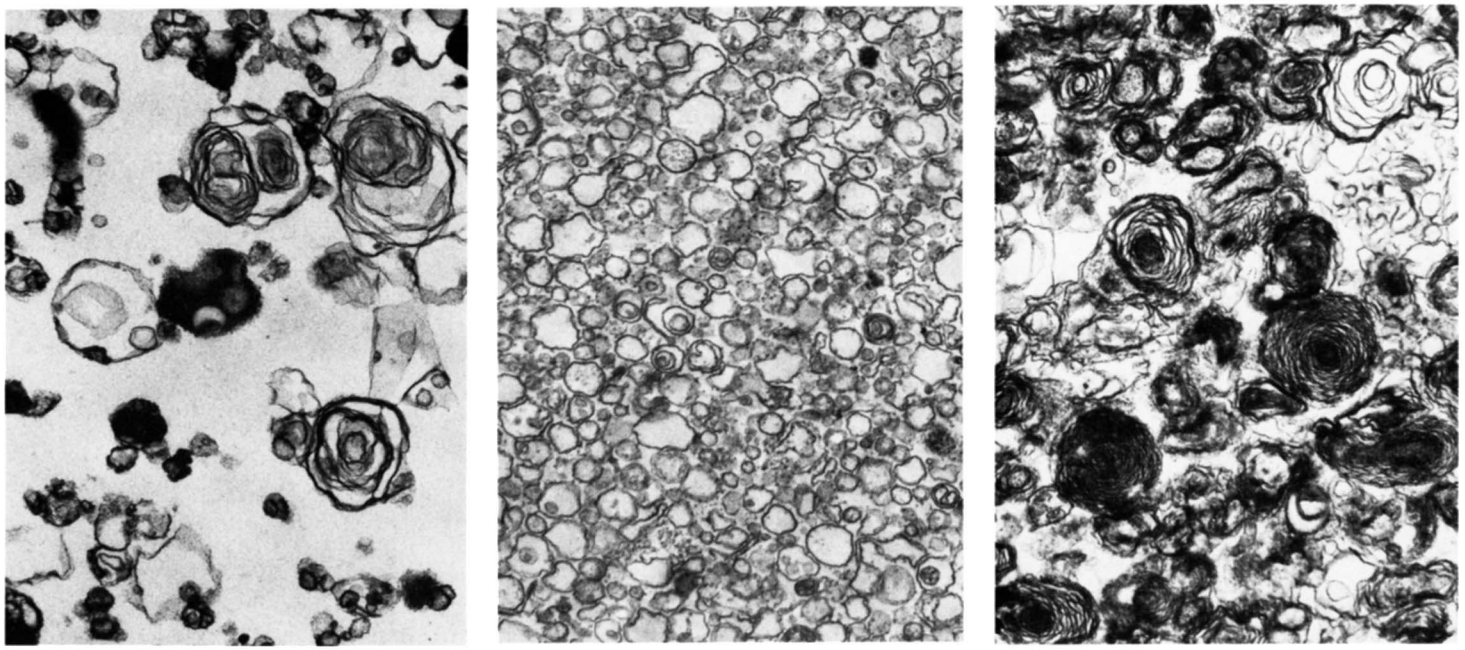

Fig. 2. Ultrastructure of purified lysosomal fractions isolated from chloroquine or DH-treated rat liver. (×20,000). Upper panel, chloroquine lysosomes I. Middle panel, chloroquine lysosomes II. Lower panel, DH lysosomes.

multilamellar bodies and a few smooth, single lamellar vesicles. Some of the multilamellar bodies had amorphous, electron-dense material in the center. The chloroquine lysosome II fraction (middle panel)

TABLE 2. Phospholipid class composition in subcellular fractions[a]

	Homogenate				Pure Mitochondria				Microsomes				Lysosomes			
	C	TWR	CHL	DH	C	TWR	CHL	DH	C	TWR	CHL	DH	TWR	CHL (I)	CHL (II)	DH
Acylphosphatidylglycerol	n.d.	0.10	1.77	0.21	n.d.	n.d.	n.d.	0.08	n.d.	0.22	n.d.	0.23	4.90	1.47	1.10	1.43
Bis(monoacylglycero)P	0.32	0.97	4.66	4.53	0.54	0.71	1.43	0.74	n.d.	0.14	1.59	1.33	23.4	13.9	10.2	9.90
Diphosphatidylglycerol	6.25	5.07	3.60	4.44	19.3	16.3	14.6	14.0	0.69	0.68	0.60	0.68	n.d.	1.00	3.02	1.59
Phosphatidylglycerol	0.22	0.57	1.09	0.76	0.43	0.68	0.55	0.80	0.05	0.46	1.23	0.17	n.d.	0.49	0.43	0.54
Phosphatidylethanolamine	26.3	24.4	21.1	19.3	35.7	35.1	30.6	32.2	23.3	23.1	21.0	21.4	11.9	12.0	14.6	10.9
Phosphatidylcholine	46.0	48.2	49.2	47.2	34.7	36.6	41.1	39.8	54.7	52.8	57.4	55.4	30.5	42.3	46.8	46.1
Phosphatidylinositol	9.88	9.56	10.6	11.7	6.54	5.78	5.35	5.63	12.8	11.9	8.74	10.44	3.95	15.7	11.3	15.0
Phosphatidylserine	3.19	3.05	3.99	3.95	0.60	1.11	1.15	1.52	2.59	3.62	4.56	3.62	3.27	7.57	5.13	4.85
Sphingomyelin	3.92	4.27	2.36	3.56	0.23	0.98	0.20	1.18	3.20	5.08	2.59	3.64	17.87	1.66	3.23	2.84
Lysophosphatidylcholine	0.86	0.87	1.57	1.57	0.46	0.55	1.93	1.42	0.72	0.59	1.13	1.55	2.23	3.08	3.51	1.97
Others	2.51	2.20	0.06	2.55	1.46	2.66	1.13	2.28	1.65	1.84	1.02	1.49	1.55	0.90	0.35	4.63
Origin	0.56	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.40	0.26	n.d.	0.08	0.04	0.54	n.d.	0.15	0.29

[a] Percent of Total Lipid Phosphorus.
Abbreviations: C, control; TWR, Triton WR-1339-treated; CHL, chloroquine-treated; DH, DH-treated; n.d., not detected.

did not contain the multilamellar bodies found in fraction I. Lysosome fraction II was composed of single lamellar vesicles with electron lucent centers. This fraction also contained some vesicles which have the appearance of rough endoplasmic reticulum. DH lysosomes (lower panel) were composed of a mixture of smooth, single lamellar vesicles with electron lucent centers, multilamellar vesicles and some electron dense bodies. These three fractions did not contain recognizable mitochondria. The respective mitochondrial fractions were free of the multilamellar bodies and smooth vesicles found in the lysosomal fractions (not shown).

Phospholipid composition of subcellular fractions

The percent phospholipid composition of the respective homogenates, microsomes, gradient-purified lysosomes and mitochondria was determined by two-dimensional thin-layer chromatography and the results are shown in **Table 2.** In the homogenate, the percent bis(monoacylglycero)phosphate was 0.32; treatment with Triton WR-1339 resulted in a 3-fold increase but treatment with chloroquine or DH resulted in a 14-fold increase in the homogenate percentage of bis(monoacylglycero)phosphate. These results are in general agreement with the data of Wherrett and Huterer for Triton WR-1339 (10), of Yamamoto et al. (5) for chloroquine and DH, and Tjiong, Lepthin, and Debuch (21) for chloroquine. Slight decreases in the percentage diphosphatidylglycerol and phosphatidylethanolamine were also observed in agreement with previous workers (4, 5).[3] In addition, treatment with chloroquine or DH was associated with a modest increase in the percent phosphatidylglycerol.[4]

Purified lysosomes from Triton-treated or drug-treated rat liver were characterized by the presence of substantial percentages of bis(monoacylglycero)phosphate and acylphosphatidylglycerol, a related compound, which comprised 10–23% and 1.1–4.9% of total lipid phosphorus, respectively. The percentage of bis(monoacylglycero)phosphate in microsomes and purified mitochondria was generally much lower than that of the homogenate. Triton lysosomes differed remarkably from chloroquine or DH lysosomes in that the tritosomes contained 17.8% sphingomyelin versus percentages of sphingomyelin of only 1.6 to 3.2% for the drug-induced lysosomes. In addition, the chloroquine and DH lysosomes had very high percentages of phosphatidylinositol, ranging from 11.2–15.6%, versus only 3.9% phosphatidylinositol in Triton lysosomes.

In contrast to lysosomes, the respective treatments had little apparent effect on the phospholipid composition of mitochondria and microsomes. As shown in Table 2, mitochondria were characterized by a high percentage of diphosphatidylglycerol (13.9–19.3%) and a low percentage of bis(monoacylglycero)phosphate (0.54–1.43%) and sphingomyelin (0.02–1.1%). Microsomes had higher percentages of sphingomyelin (2.5–5.0%) than that of mitochondria, and contained very little diphosphatidylglycerol or bis(monoacylglycero)phosphate. These data confirm the importance of bis(monoacylglycero)phosphate as a specific marker lipid for secondary lysosomes.

Content per mg protein of total phospholipid, bis(monoacylglycero)phosphate and phosphatidylinositol in subcellular fractions

The content of total phospholipids, bis(monoacylglycero)phosphate, and phosphatidylinositol per mg protein of the subcellular fraction is shown in **Table 3.** Triton WR-1339 had no effect on the total phospholipid content of the homogenate. However, homogenate phospholipid content increased from 207 to 320 with chloroquine and to 307 with DH. The gradient-purified mitochondria have a normal phospholipid content. Lysosomes purified from the M fraction of drug-treated rat liver have an extremely high content of lipid phosphorus per mg protein; chloroquine lysosomes I, 1765 nmol per mg protein; chloroquine lysosomes II, 607 nmol per mg protein, and DH lysosomes, 2612 nmol per mg protein. From the phospholipid to protein ratio (Table 3), the percent of protein in the M fraction (Fig. 1) and the percent of recovered protein in each of the purified components of the M fraction, the contribution of lysosomes and mitochondria to the phospholipid content of the M fraction can be calculated. With chloroquine treatment, lysosomes I and II and mitochondria accounted for 77, 21, and 50 μmol lipid phosphorus per fraction, respectively, where the homogenate has been set to 1.0 g protein. With DH treatment, lysosomes and mitochondria account for 102 and 50 μmol lipid phosphorus, respectively, while control mitochondria account for about 55 μmol of lipid phosphorus. Thus,

[3] For simplicity, Table 2 shows the results of single experiments only. However, in other experiments (data not shown), the following additional data were obtained and the means and standard deviations calculated. The figures in parentheses represent the number of replicates. Percentage diphosphatidylglycerol in the homogenate: control, 6.0 ± 0.3 (9); chloroquine, 4.6 ± 0.6 (5); and DH, 4.3 ± 0.3 (5); $P < 0.02$ for both drugs versus the control, respectively. Percentage phosphatidylethanolamine in the homogenate: control, 26.5 ± 1.0 (9); chloroquine, 19.6 ± 1.5 (5); and DH, 19.2 ± 0.3 (5). $P < 0.01$ and < 0.001, respectively, versus the control.

[4] The percentage phosphatidylglycerol in the various homogenates was as follows: control, 0.46 ± 0.14 (9); chloroquine, 1.19 ± 0.22 (5) and DH, 1.24 ± 0.46 (5). $P < 0.01$ and < 0.02, respectively, versus the control.

TABLE 3. Content of total phospholipids, bis(monoacylglycero)P,

	Total Phospholipids[a]				Bis(monoacylglycero)P[a]				Phosphatidylinositol[a]			
	C	TWR	CHL	DH	C	TWR	CHL	DH	C	TWR	CHL	DH
Homogenate (H)	207	193	320	307	0.7	1.9	14.9	13.9	22.5	16.5	34.2	34.1
Nuclei, debris (N)	205	233	224	272	0.8	0.7	11.1	6.5	17.1	19.2	17.8	24.5
Heavy mitochondria (M)	255	241	522	512	2.5	5.2	38.0	27.5	20.5	18.6	55.6	60.9
Light mitochondria (L)	451	399	439	651	2.9	5.7	10.2	23.1	57.6	43.7	42.2	74.1
Microsomes (P)	639	480	584	608	n.d.	0.6	9.3	4.0	82.1	56.9	51.0	63.4
Supernatant (S)	16	22	54	72	0.1	1.1	1.2	2.7	1.9	1.3	4.7	9.0
Pure mitochondria	213	199	236	224	1.2	1.4	3.4	1.7	13.9	11.5	12.6	12.6
Lysosomes I		387	1770	2610		90.3	243	259		15.3	276	391
Lysosomes II			607				61.7				68.4	

[a] μmol per g protein.
[b] Desmosterol, μmol per g protein.
Abbreviations: C, control; TWR, Triton WR-1339; CHL, chloroquine; DH, 4,4′-bis(β-diethylaminoethoxy)α,β-diethyldiphenylethane; numbers in parentheses represent desmosterol; n.d., not detected.

the entire increase in liver phospholipid with chloroquine or DH treatment, 113 and 100 μmol lipid phosphorus per 1.0 g protein, can be accounted for by the phospholipid content of the respective lysosomal fractions.

Bis(monoacylglycero)phosphate increased to a much greater degree than did total phospholipids (Table 3). Bis(monoacylglycero)phosphate in the homogenate, the heavy mitochondria (M), and the light mitochondria (L) of Triton WR-1339-treated rat showed a two- or three-fold increase over the respective fractions from the untreated control rats. The increase in these fractions was apparently due to the presence of secondary lysosomes which have a very high bis(monoacylglycero)phosphate content, 90.3 nmol per mg protein. In chloroquine and DH-treated rat liver, the homogenate bis(monoacylglycero)phosphate content was increased more than twenty-fold over the control. Bis(monoacylglycero)-phosphate was higher in every fraction as compared with control rat liver but the increase was the most remarkable in the heavy mitochondria (M). The highest content of bis(monoacylglycero)phosphate was found in the pure lysosomal fraction of the drug-treated rat liver: chloroquine lysosomes I, 243.6 nmol per mg protein; chloroquine lysosomes II, 61.7 nmol per mg protein; and DH lysosomes, 258.6 nmol per mg protein. Other membranes such as mitochondria and microsomes had a very low content of bis(monoacylglycero)phosphate which can be accounted for by slight contamination of these fractions with lysosomes (Table 1).

The phosphatidylglycerol content of the chloroquine and DH homogenates was 5- to 6-fold greater than that of control homogenates (calculated from data in Tables 1 and 2). The homogenate content of phosphatidylcholine and phosphatidylethanolamine was 1.4- to 1.7-fold greater than control. It is clear that phosphatidylglycerol and bis(monoacylglycero)phosphate increase to a much greater degree (5- and 20-fold, respectively) than the major phosphoglycerides (1.4- to 1.7-fold).

The phosphatidylinositol content of subcellular fractions is shown in Table 3. In Triton WR-1339-treated rat liver, the phosphatidylinositol content was slightly decreased in the homogenate and in the microsomes but was essentially unchanged in other subcellular fractions. However, the phosphatidylinositol content of the liver homogenate of drug-treated rats was increased 1.7- and 1.8-fold above that of the control. The increase in cellular phosphatidylinositol can also be explained by the presence of the phospholipid-rich lysosomes since the phosphatidylinositol content of purified mitochondria and microsomes was not increased. The purified lysosomes were found to have remarkably high phosphatidylinositol content, 276 to 391 nmol per mg protein, 5 times higher than that of the microsomes and 20 to 30 times greater than that of mitochondria. When analyzed on the basis of lipid content per mg protein, the low phosphatidylinositol content of Triton lysosomes is again apparent, 15.3 nmol per mg. Thus, the chloroquine and DH lysosomes are 18–25 times richer in phosphatidylinositol than are Triton lysosomes.

Cholesterol content of the subcellular fractions

The free and esterified cholesterol content of the respective subcellular fractions is shown in Table 3. In the respective homogenates, free cholesterol was increased above that of the control only in Triton-treated rat liver. Desmosterol was detected in the homogenate and subcellular fractions from the liver of DH-treated rats, but was not detected in the other preparation in agreement with previous studies (1). In the liver of Triton WR-1339-, chloroquine-, and DH-treated rats, the cholesteryl ester content per mg

phosphatidylinositol, cholesterol, and drugs in subcellular fractions

Free Cholesterol[a]				Esterified Cholesterol[a]				Drug Content[a]	
C	TWR	CHL	DH	C	TWR	CHL	DH	CHL	DH
22.5	33.3	19.5	25.2 (2.9[b])	2.2	30.0	11.8	12.9 (3.3[b])	28.2	17.3
19.5	30.3	15.2	22.8 (2.3)	0.8	10.0	0.2	3.7 (0.5)	9.6	10.6
19.6	25.9	22.5	33.6 (5.9)	0.5	22.4	20.1	11.9 (3.6)	94.2	44.5
67.8	80.9	30.3	56.7 (8.4)	8.0	70.6	34.9	24.8 (10.1)	17.5	38.1
70.0	97.7	44.6	60.2 (4.5)	1.4	33.4	17.4	10.2 (7.4)	11.3	6.5
2.1	11.6	0.26	7.2 (1.5)	4.8	28.2	9.0	6.5 (4.3)	n.d.	3.5
1.5	5.8	1.6	5.8 (0.4)	1.1	1.1	0.9	0.02 (0.02)	8.6	2.8
	167.9	58.8	293 (21.3)		133	42.2	39.4 (37.1)	384	189
		61.2				19.4		55.3	

protein in the homogenate was greatly increased over that of control rats. With chloroquine and DH treatment, the increased phospholipid content can be accounted for by the presence of the secondary lysosomes and multilamellar bodies. However the esterified cholesterol is present in increased amounts in many subcellular sites including the heavy and light mitochondrial fractions, microsomes, lysosomes, and supernatant. Esterified desmosterol was present in highest concentration in DH-treated liver microsomes. 7.4 nmol per mg; light mitochondria, 10.1 nmol per mg, and lysosomes, 37.1 nmol per mg. Finally, it should be noted that our data on the free and esterified cholesterol content of normal liver mitochondria and microsomes are in agreement with the results of other workers (34).

Distribution of chloroquine and DH in the subcellular fractions

The concentration of chloroquine and DH per mg protein is shown in Table 3. Several metabolic products of DH were also detected, but the data in Table 3 represent the sum of DH and its metabolites. The concentration of these two drugs is highest in the heavy and light mitochondrial fractions although a small amount is also present in microsomes and purified mitochondria. The concentration of DH in the supernatant fraction was very low; surprisingly, chloroquine was not detected in the supernatant fraction. The multilamellar bodies isolated from the heavy mitochondrial fraction had a chloroquine content relative to protein which was 13.5-fold greater than that of the homogenate; the multilamellar body fraction from DH-treated liver had a 10.9-fold greater DH content than the homogenate. Thus, these experiments provide direct proof of the marked accumulation of chloroquine and DH in lysosomes. In view of the low levels of chloroquine and DH in the supernatant fractions and the fact that drugs are retained in the lysosomal fractions even after extensive manipulations including centrifugation and recovery from sucrose gradients, it seems reasonable to infer that there is a very tight association between the respective drugs and the lysosomes.

DISCUSSION

Oral administration of chloroquine or DH to rats for 1 week (100 mg/kg) resulted in a 50% increase in liver phospholipid content. Our findings are in agreement with the previous experiments of Yamamoto et al. (5) but differ from the results of Tjiong et al. (21) who found no increase in liver phospholipid content with DH and only a 10% increase with chloroquine treatment, respectively. This is due in all likelihood to differences in drug dosage (100 mg/kg versus 10–60 mg/kg). However, our results indicating that chloroquine- or DH-induced lysosomes are highly enriched in phospholipids and bis(monoacylglycero)phosphate confirm the data of Tjiong et al. (21, 22). In addition, the data presented in this paper, which are supported by marker enzymes, make it clear that the secondary lysosome is the sole intracellular site of bis(monoacylglycero)phosphate.

Analysis of the subcellular distribution of lipids in the liver of rats treated with these drugs indicates that the excess phospholipid content of drug-treated liver can be completely explained by the presence of these phospholipid-rich secondary lysosomes which are the intracellular site of bis(monoacylglycero)-phosphate. We have also shown that the secondary lysosomes contain the enzyme required for the synthesis of bis(monoacylglycero)phosphate. Finally, our studies give direct evidence of the accumulation of chloroquine and DH in the lysosomes. DH has not previously been reported to concentrate in lysosomes while several groups have shown chloroquine accumulation in lysosomes (9).

The lipid composition of the respective drug-induced lysosomes (21, this paper) is considerably different than that of the membranous cytoplasmic

bodies (MCB) of Niemann-Pick disease, a hereditary sphingolipidosis. In Niemann-Pick disease, the accumulation of sphingomyelin, bis(monoacylglycero)-phosphate and free cholesterol is striking and the molar ratio of cholesterol to lipid phosphorus in the MCB fraction is 0.30 (35). However, the ratio of cholesterol to phospholipid in these drug-induced multilamellar bodies is much lower, ranging from 0.05 to 0.13. The similarity in the ratio of the sum of the drug plus cholesterol to phospholipid (0.27, 0.22, 0.20 in chloroquine lysosome I, chloroquine lysosome II, and DH lysosome, respectively) to the cholesterol/phospholipid ratio of the Niemann-Pick MCB (0.30) suggests the possibility that the respective drugs have replaced free cholesterol as a membrane component in the drug-induced multilamellar bodies.

The marked accumulation of cholesterol ester in the liver is also a consequence of treatment with chloroquine, DH and Triton WR-1339 (Table 3). Homogenate cholesterol ester is increased 5.4-, 5.9-, and 73.6-fold with chloroquine, DH, and Triton WR 1339, respectively. With chloroquine and DH treatment, the excess cholesterol ester is found predominantly in the M and L fractions, accounting for 55–60% of cellular cholesterol ester versus only 14% present in the M and L fractions of the control rat liver while with Triton WR-1339 treatment, 37% of the esterified cholesterol is present in the M and L fractions. Microsomal and supernatant cholesteryl ester content is also increased by treatment with Triton WR 1339, chloroquine and DH. In contrast to phospholipid accumulation caused by the two drugs which is confined to lysosomes, cholesterol ester accumulation occurs in several intracellular sites including microsomes, lysosomes, and the supernatant fraction. Our data with chloroquine are in close agreement with the intracellular distribution of radioactive cholesteryl ester in the liver of chloroquine-treated rats reported by Floren, Nordgren, and Nilsson (36). These results are compatible with the hypothesis that treatment with chloroquine or DH blocks the lysosomal degradation of cholesteryl esters. However, increased esterification of cholesterol by acyl CoA:cholesterol acyltransferase is also likely in view of the increased microsomal content of cholesteryl ester.

What is the molecular basis for the accumulation of phospholipid in multilamellar bodies? First, phospholipid reaches the lysosomes either by the uptake of extracellular material such as lipoproteins which enter the cell by adsorptive endocytosis (37) or surface transport (38) or by interaction with intracellular membranes which contain phospholipid (autophagy). Ultrastructural examination of liver tissue following treatment with chloroquine or DH shows ample evidence for increased autophagy (14–17), and it seems likely that some of the phospholipid which accumulates in lysosomes is of intracellular origin. A contribution of lipoprotein phospholipid to the storage condition is also probable since both low and high density lipoprotein are taken up by rat liver (39, 40). At the present time it is difficult to assess the relative importance of the contribution of these two sources of lipid. Nevertheless, it seems reasonable to assume that increased transfer of phospholipids to lysosomes is an important factor in the mechanism of drug-induced phospholipidosis.

Secondly, phospholipid degradation by lysosomal acid phospholipase may be impaired. Chloroquine has a well-known tendency to concentrate on lysosomes where it might inhibit acid phospholipase A due to its ability to raise the intralysosomal pH (8, 9). Inhibition of the catabolism of proteins and cholesteryl ester in liver by chloroquine has been reported previously (6, 7, 36). It has also been proposed that cationic amphiphilic agents bind tightly to phospholipids forming complexes which may be resistant to hydrolysis by phospholipase A (19). However, this theory has not yet been tested directly with lysosomal acid phospholipase A. Although our studies have shown for the first time that DH is concentrated in lysosomes, its effects on lysosomal acid hydrolases, phospholipases, and the intralysosomal pH are as yet unknown and remain to be investigated.

The high content of bis(monoacylglycero)phosphate and phosphatidylinositol in multilamellar bodies deserves further comment. Our previous studies have shown that bis(monoacylglycero)phosphate is synthesized in lysosomes from phosphatidylglycerol or lysophosphatidylglycerol (26, 41). In this reaction, an acyl group from phosphatidylinositol is transferred to the glycerol-1-P moiety of phosphatidylglycerol or lysophosphatidylglycerol (42). The high concentration of phosphatidylinositol in the lysosomes induced by chloroquine or DH may be partly responsible for the accumulation of bis(monoacylglycero)phosphate due to its role as a substrate in the biosynthesis of bis(monoacylglycero)phosphate (42). Once formed, bis(monoacylglycero)phosphate would be more prone to accumulate than other phosphoglycerides, since this phosphoglyceride is known to be resistant to degradation by lysosomal hydrolases. We recently found that the initial rate of bis(monoacylglycero)-phosphate degradation by lysosomal acid hydrolases is only 10% of that of dioleoylphosphatidylcholine (43). This may be due to its unusual *sn*-1-glycerophospho-*sn*-1′-glycerol stereoconfiguration (44), and to its participation in a futile cycle which tends to reform bis(monoacylglycero)phosphate (43). In addition,

phosphatidylinositol and other negatively-charged phospholipids might form stable complexes with cationic amphiphilic agents thus protecting the acidic phospholipids from hydrolysis by lysosomal phospholipases to a greater degree than neutral phosphoglycerides. Finally, it is possible that phosphatidylinositol and phosphatidylglycerol, the precursors of bis(monoacylglycero)phosphate, might be overproduced. It has been suggested that cationic amphiphilic drugs affect lipid metabolism by inhibiting phosphatidate phosphohydrolase, leading to the overproduction of CDP-diacylglycerol and its metabolic products, phosphatidylinositol and phosphatidylglycerol (45). The overproduction of these phospholipids, the substrates for bis(monoacylglycero)phosphate biosynthesis, would be expected to lead to accelerated formation of this lysosomal lipid (42). However, further studies are needed to delineate the molecular mechanisms of this unusual toxic effect of cationic amphiphilic drugs.

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REFERENCES

1. Yamamoto, A., S. Adachi, K. Ishikawa, T. Yokomura, T. Kitani, T. Nasu, T. Imoto, and M. Nishikawa. 1971. Studies on drug-induced lipidosis III. Lipid composition of the liver and some other tissues in clinical cases of "Niemann-Pick-like Syndrome" induced by 4,4′-diethylaminoethoxyhexestrol. *J. Biochem. (Tokyo)* **70:** 775–784.
2. Yamamoto, A., S. Adachi, T. Kitani, Y. Shinji, K. Seki, T. Nasu, and M. Nishikawa. 1971. Drug-induced lipidosis in human cases and in animal experiments: Accumulation of an acidic glycerophospholipid. *J. Biochem. (Tokyo)* **69:** 613–615.
3. Matsuzawa, Y., A. Yamamoto, S. Adachi, and M. Nishikawa. 1977. Studies in drug-induced lipidosis VIII. Correlation between drug accumulation and acidic phospholipids. *J. Biochem. (Tokyo)* **82:** 1369–1377.
4. Adachi, S., Y. Matsuzawa, T. Yokomura, K. Ishikawa, S. Uhara, A. Yamamoto, and M. Nishikawa. 1972. Studies on drug-induced lipidosis (V). Changes in the lipid composition of rat liver and spleen following the administration of 4,4′-diethylaminoethoxyhexestrol. *Lipids.* **7:** 1–7.
5. Yamamoto, A., S. Adachi, Y. Matsuzawa, T. Kitani, A. Hiraoka, and K. Seki. 1976. Studies on drug-induced lipidosis: VII. Effects of bis-β-diethylaminoethylether of hexestrol, chloroquine, homochlorocyclizine, prenylamine and diazacholesterol on the lipid composition of rat liver and kidney. *Lipids.* **11:** 616–622.
6. Goldstein, J. L., G. Y. Brunschede, and M. S. Brown. 1975. Inhibition of the proteolytic degradation of low density lipoprotein in human fibroblasts by chloroquine, concanavalin A, and Triton WR-1339. *J. Biol. Chem.* **250:** 7854–7862.
7. Stein, Y., V. Ebin, H. Bar-On, and O. Stein. 1977. Chloroquine-induced interference with degradation of serum lipoproteins in rat liver, studied in vivo and in vitro. *Biochim. Biophys. Acta.* **486:** 286–297.
8. Ohkuma, S., and B. Poole. 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Nat. Acad. Sci. USA.* **75:** 3327–3331.
9. de Duve, C., T. de Barsy, B. Poole, A. Trouet, P. Tulkens, and F. van Hoof. 1974. Commentary: Lysosomotropic agents. *Biochem. Pharmacol.* **23:** 2495–2531.
10. Wherret, J. R., and S. Huterer. 1972. Enrichment of bis(monoacylglyceryl)phosphate in lysosomes from rat liver. *J. Biol. Chem.* **247:** 4114–4120.
11. Brotherus, J., and O. Renkonen. 1977. Subcellular distributions of lipids in cultured BHK cells: evidence for the enrichment of lysobisphosphatidic acid and neutral lipids in lysosomes. *J. Lipid Res.* **18:** 191–202.
12. Brotherus, J., and O. Renkonen. 1977. Phospholipids of subcellular organelles isolated from cultured BHK cells. *Biochim. Biophys. Acta.* **486:** 243–253.
13. Mason, R. J., T. P. Stossel, and M. Vaughan. 1972. Lipids of alveolar macrophages, polymorphonuclear leukocytes, and their phagocytic vesicles. *J. Clin. Invest.* **51:** 2399–2497.
14. Seki, K., Y. Simji, and M. Nishikawa. 1971. Studies on drug-induced lipidosis II. Light and electron microscopic observations on the liver biopsy specimens. *Acta Hepatol. Jap.* **12:** 226–232.
15. de la Iglesia, F. A., G. Feuer, A. Takada, and Y. Matsuda. 1974. Morphologic studies on secondary phospholipidosis in human liver. *Lab. Invest.* **30:** 539–549.
16. Abraham, R., R. Hendy, and P. Grasso. 1968. Formation of myeloid bodies in rat liver lysosomes after chloroquine administration. *Exp. Mol. Pathol.* **9:** 212–229.
17. Tashiro, Y. 1975. An electron microscopic observation on the cytological changes in experimental drug-induced lipidosis. *Keio J. Med.* **24:** 115–143.
18. Lüllmann, H., R. Lüllmann-Rauch, and O. Wassermann. 1973. Drug-induced phospholipidosis. *Ger. Med.* **3:** 128–135.
19. Seydel, J. K., and O. Wasserman. 1976. NMR studies on the molecular basis of drug-induced phospholipidosis. II. Interaction between several amphiphilic drugs and phospholipids. *Biochem. Pharmacol.* **25:** 2357–2364.
20. Sarzala, M. G., L. M. G. von Golde, B. De Kruff, and L. L. M. van Deenen. 1970. The intramitochondrial distribution of some enzymes involved in the biosynthesis of rat-liver phospholipids. *Biochim. Biophys. Acta.* **202:** 106–119.
21. Tjiong, H. B., J. Lepthin, and H. Debuch. 1978. Lysosomal phospholipids from rat liver after treatment with different drugs. *Hoppe-Seyler's Z. Physiol. Chem.* **359:** 63–69.
22. Tjiong, H. B., and H. Debuch. 1978. Lysosomal bis-(monoacylglycero)phosphate of rat liver, its induction by chloroquine and its structure. *Hoppe-Seyler's Z. Physiol. Chem.* **359:** 71–79.

23. Trouet, A. 1974. Isolation of modified liver lysosomes, *In* Methods in Enzymology, Vol. 31, Biomembranes, Part A., S. Fleisher and L. Packer, editors. Academic Press, New York. 323–329.
24. Green, D. W., S. Mii, and P. M. Kohout. 1955. Studies on the terminal electron transport system. I. Succinic dehydrogenase. *J. Biol. Chem.* **217:** 551–567.
25. Sottacasa, G. L., B. Kuylenstierna, L. Ernster, and A. Bergstrand. 1967. An electron transport system associated with the outer membrane of liver mitochondria. *J. Cell Biol.* **32:** 415–438.
26. Poorthuis, B. J. H. M., and K. Y. Hostetler. 1976. Studies on the subcellular localization and properties of bis-(monoacylglyceryl)phosphate biosynthesis in rat liver. *J. Biol. Chem.* **251:** 4596–4602.
27. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple procedure for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
28. Cotman, C. W., and D. A. Flansburg. 1970. An analytical micro-method of electron microscopic study of the composition and sedimentation properties of subcellular fractions. *Brain Res.* **22:** 152–156.
29. Poorthuis, B. J. H. M., P. J. Yazaki, and K. Y. Hostetler. 1976. An improved two-dimensional thin-layer chromatography system for the system for the separation of phosphatidylglycerol and its derivatives. *J. Lipid Res.* **17:** 433–437.
30. Rouser, G., S. Fleischer, and A. Yamamoto. 1969. Two-dimensional thin-layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids.* **5:** 494–496.
31. Ishikawa, T. T., J. MacGee, J. A. Morrison, and C. J. Glueck. 1974. Quantitative analysis of cholesterol in 5 to 20μl of plasma. *J. Lipid Res.* **15:** 286–291.
32. de Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans. 1955. Intracellular distribution patterns of enzymes in rat liver tissue: *Biochem. J.* **60:** 604–617.
33. Plattner, H., R. Henning, and B. Brauser. 1975. Formation of Triton WR-1339-filled rat liver lysosomes. II. Involvement of autophagy and of pre-existing lysosomes. *Exp. Cell. Res.* **94:** 377–391.
34. Zambrano, F., S. Fleischer, and B. Fleischer. 1975. Lipid composition of the Golgi apparatus of rat kidney and liver in comparison with other subcellular organelles. *Biochim. Biophys. Acta.* **380:** 357–369.
35. Kamoshita, S., A. M. Aron, K. Suzuki, and K. Suzuki. 1969. Infantile Niemann-Pick disease. A chemical study with isolation and characterization of membranous cytoplasmic bodies and myelin. *Amer. J. Dis. Child.* **117:** 379–394.
36. Floren, C. H., H. Nordgren, and A. Nilsson. 1977. Effect of chloroquine and colchicine on the degradation of chyle cholesteryl ester and phospholipids in vivo. *Eur. J. Biochem.* **80:** 331–340.
37. Goldstein, J. L., and M. S. Brown. 1977. The low-density lipoprotein pathway and its relation to atherosclerosis. *Ann. Rev. Biochem.* **46:** 897–930.
38. Rothblat, G. H., L. A. Arbogast, and E. K. Ray. 1978. Stimulation of esterified cholesterol accumulation in tissue culture cells exposed to high density lipoproteins enriched in free cholesterol. *J. Lipid Res.* **19:** 350–358.
39. Sigurdsson, G., S-P. Noel, and R. J. Havel. 1978. Catabolism of the apoprotein of low density lipoprotein by the isolated perfused rat liver. *J. Lipid Res.* **19:** 628–634.
40. Sigurdsson, G., S-P. Noel, and R. J. Havel. 1979. Quantification of the hepatic contribution to the catabolism of high density lipoprotein. *J. Lipid Res.* **20:** 316–324.
41. Poorthuis, B. J. H. M., and K. Y. Hostetler. 1978. Conversion of diphosphatidylglycerol to bis(monoacylglycero)phosphate by lysosomes. *J. Lipid Res.* **19:** 309–315.
42. Matsuzawa, Y., B. J. H. M. Poorthuis, and K. Y. Hostetler. 1978. Mechanism of phosphatidylinositol stimulation of lysosomal bis(monoacylglyceryl)phosphate synthesis. *J. Biol. Chem.* **253:** 6650–6653.
43. Matsuzawa, Y., and K. Y. Hostetler. 1979. Degradation of bis(monoacylglycero)phosphate by an acid phosphodiesterase in rat liver lysosomes. *J. Biol. Chem.* **254:** 5997–6001.
44. Joutti, A., J. Brotherus, O. Renkonen, R. Laine, and W. Fischer. 1976. The stereochemical configuration of lysobisphosphatidic acid from rat liver, rabbit lung and pig lung. *Biochim. Biophys. Acta.* **450:** 206–209.
45. Brindley, D. N., and M. Bowley. 1975. Drug affecting the synthesis of glycerides and phospholipids in rat liver. *Biochem. J.* **148:** 461–469.